Aspects of the ecology and population dynamics of the fungus *Beauveria bassiana* strain F418 in soil

---

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

At
Lincoln University
By
Céline Blond

Lincoln University
2012

Abstract

Abstract of a thesis submitted in partial fulfilment of the
requirements for the Degree of Doctor of Philosophy.

Aspects of the ecology and population dynamics of the fungus *Beauveria bassiana* strain F418 in soil

by

Céline Blond

This research aimed to improve understanding of the ecology of *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales) strain F418 in soil, aided by the use of *gfp* transformants, to improve the use of this fungus as a biopesticide in New Zealand pastures.

Prior to using *B. bassiana* F418 *gfp* transformants (F418 *gfp* tr1 and F418 *gfp* tr3), their phenotypes were comprehensively compared to the wild-type F418. Compared to F418, F418 *gfp* tr3 had a faster rate of germination at 15 °C for 24 h and 20 °C for 14 h and F418 *gfp* tr1 had a slower rate of germination at 25 °C for 14 h; however this was not apparent at longer incubation times at all temperatures. F418 *gfp* tr3 colony growth rate was slower than F418 at 15 and 20 °C but it was not different at 25 °C. There was no difference in conidial production and conidia size at different incubation temperatures. There were no differences in virulence against *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae and conidia production on cadavers using different fungal concentrations, *T. molitor* larval size or using either single or mixed fungal strains. F418 *gfp* tr1 phenotypical characteristics were more similar to F418 than F418 *gfp* tr3, however, F418 *gfp* tr3 fluorescence was substantially brighter than F418 *gfp* tr1. Therefore, both transformed strains could be used as substitutes for the wild-type in ecological studies. In an effort to explain the observed differences, an attempt was made to find the insertion point of the *gfp* cassette inside the transformant genomes.

F418 persistence was initially assessed in microcosms of pasteurised soil under eight different abiotic, biotic and pre-application factors. F418 persistence was better with (i) low water content (14 and 22 % gravimetric water content) compared to high water content (31 % gravimetric water content), (ii) soil at 10 and 15 °C compared to 20 °C, (iii) soil containing 7.5 % peat compared to 0 and 15 % peat and (iv) superphosphate fertiliser than with nitrogen. Persistence of F418 was lower in the presence of non-host insects than in soil without insects. Generally there was an increase in F418 populations in the first few weeks in

pasteurised soil. The results using the transformants with different moisture levels, fertilisers and the presence or absence of insects were similar to those observed for F418 in pasteurised soil but the findings were different in non-sterile soil. Moisture levels and presence of host, non-host and no insects were chosen as key factors warranting further investigation. F418 *gfp* tr3 was used to study the effect of these two factors more intensively in pasteurised and non-sterile soil.

This study reports the development of a modified soil sandwich method to assess fungal propagule dynamics (conidia *vs.* mycelia). Results from the moisture experiments showed that the increase of F418 and F418 *gfp* tr3 observed in the first few weeks in pasteurised soil was likely due to the development of mycelia. Soil communities assessed using single strand conformation polymorphism (SSCP) showed a correlation with F418 *gfp* tr3 persistence in pasteurised and non-sterile soil. The lower F418 *gfp* tr3 populations observed in pasteurised soil with a high moisture level may have been due to antagonistic microbes.

F418 *gfp* tr3 populations were lower in pasteurised soil in the presence of non-host *Costelytra zealandica* White (Coleoptera: Scarabaeidae) larvae than without insects. Further investigations showed that F418 *gfp* tr3 could attach, germinate and form appressoria on *C. zealandica* cuticle. Conidia of *C. zealandica* larvae were shown to survive passage through the *C. zealandica* gut. Differences in the soil communities, as assessed by SSCP, correlated with differences in persistence in pasteurised and non-sterile soil with host, non-host and without insects. The lower F418 *gfp* tr3 populations observed in pasteurised soil with *C. zealandica* could have been due to non-specific germination on non-host cuticle and/or presence of antagonistic microbes.

F418 *gfp* tr3 interactions with red clover plants were studied in the final chapter to add some complexity to the simplified microcosms and be more similar to the pasture reality. F418 *gfp* tr3 could colonise the rhizosphere in pasteurised soil but this was not observed in non-sterile soil. F418 *gfp* tr3 was found as an endophyte in red clover roots and stems after 18 weeks in pasteurised and non-sterile soil.

Overall, this research provides new insights on *B. bassiana* F418 ecology in soil and highlighted the importance of studying *B. bassiana* population dynamics in non-sterile soil where soil microorganisms have a strong impact.

Keywords: *Beauveria bassiana*, entomopathogenic fungus, soil, *gfp* transformant, persistence, soil moisture, non-host insect, *Costelytra zealandica*, clover, endophyte, population dynamics, soil sandwich.

Acknowledgements

I would like to thanks my supervisors Dr Hayley Ridgway, Prof. Travis Glare, Dr Mickael Brownbridge, Assoc. Prof. R. Bruce Chapman and Prof. Leo Condron for their invaluable advices and guidance throughout this PhD. I would not have finished this doctorate without your encouragement.

I would also like to thanks the following people for their help: Dave Saville for his statistical expertise, Norma Merrick for the sequencing, Richard Townsend for providing grass grubs, Dalin Brown for teaching me the SSCP method, Sean Marshall for showing me some techniques with grass grubs, Tracey Nelson with diverse methods in microbiology, Nic Cummings for his help with PCR and phylogenic studies, Neil Smith for the use of Equi-pf and the pressure plates, Aimee McKinnon for proof reading my thesis at the last minute, Sophie Mambrini and Clément Lignon for their help digging hundreds of kilograms of soil while reshaping Europe and particularly Sophie for spending so many hours helping me remove roots and worms, all of that for having used only a tenth of the soil.

Enfin, j'aimerai tout particulièrement remercier mes parents qui m'ont supporté pendant toutes ces années.

I would like to acknowledge the Tertiary Education Commission for their financial support.

Table of Contents

List of Tables

List of Figures

Abbreviations and Symbols

AD	arbitrary degenerate
ANOVA	analysis of variance
BCA	biological control agent
BLAST	basic local alignment search tool
bp	base pair
C	carbon
CFU	colony forming unit
cm	centimetre
C/N	carbon to nitrogen ratio
CO_2	carbon dioxide
d	day (s)
d.f.	degree of freedom
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DWS	dry weight soil
EDTA	ethylenediaminetetraacetic acid
EF1-α	elongation factor 1-α
F418	*Beauveria bassiana* strain F418
F418 *gfp* tr1	*Beauveria bassiana* strain F418 *gfp* tr1
F418 *gfp* tr3	*Beauveria bassiana* strain F418 *gfp* tr3
FC	field capacity
g	gram (s)
gfp	green fluorescent protein
GWC	gravimetric water content
h	hour (s)
H_2O	water
ha	hectare
INIL	initial inoculum level
ITS	nuclear ribosomal internal transcribed spacer region

Kb	kilo base pair
L	litre
LSD	least significant difference
m	metre
Mb	mega base pair
MDE	mutation detection enhancement
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
N	nitrogen
ng	nanogram
O_2	dioxygen
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDA+A	potato dextrose agar amended with tetracycline chloride, streptomycin sulphate and cycloheximide (Appendix A)
pg	picogram
pM	picoMole
®	registered trademark
RCBD	random complete block design
rDNA	ribosomal deoxyribonucleic acid
RH	relative humidity
RNA	ribonucleic acid
s	second
SDA	Sabouraud dextrose agar
SDW	sterile distilled water
SDWA	sterile distilled water agar
sp.	specie
spp.	Species
SSCP	single strand conformation polymorphism
TAE	tris acetate ethylenediamine tetraacetic acid

TAIL PCR	thermal asymmetric interlaced PCR
TBE	tris borate ethylenediamine tetraacetic acid
™	trademark
U	units
µg	microgram
µL	microlitre
µm	micrometre
µM	micromolar
V	volt
v/v	volume to volume ratio
w/v	weight to volume ratio
w/w	weight to weight ratio
Ø	diameter
%	percentage
°C	Celcius degree
10 % SDA	one tenth strenght Sabouraud dextrose agar (Appendix A)
10 % SDA+A	10 % SDA amended with tetracycline chloride, streptomycin sulphate and cycloheximide (Appendix A)
10 % SDA+A+Hyg	10 % SDA amended with tetracycline chloride, streptomycin sulphate and cycloheximide and hygromycin B (Appendix A)

Chapter 1
Introduction

1.1 Research background

The clover root weevil, *Sitona lepidus* Gyllenhal (Coleoptera: Curculionidae) is a major pest of white clover in New Zealand pastures. This exotic pest was first identified in 1996 in the North Island (Barratt *et al*., 1996) and has since spread to the South Island (Phillips *et al*., 2007). The larvae live in the soil and feed on the nitrogen fixating nodules, the roots and the stolons of white clover while the adult feed on the clover leaves (Gerard, 2001), therefore, reducing the clover nitrogen fixation and the forage quality of pasture for animal production. Clover root weevil larval density can reach up to 1,800 larvae/m^2 during its initial boom and bust cycle and then stabilise to a density of 450-750 larvae/m^2 (Gerard *et al*., 2010). The damage caused by the clover root weevil could cost $0.2-1 billion per year to New Zealand farmers (Wear & Andrews, 2005 cited in Phillips *et al*., 2007) due to the cost of additional nitrogen fertiliser application to balance the loss of nitrogen fixed by clover plants and the extra animal feed required to compensate for the foraging loss.

Following the discovery of this new pasture pest in New Zealand, it was hoped that the Moroccan strain of the parasitoid wasp *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae), introduced for the successful control of the lucerne weevil *Sitona discoideus* Gyllenhal (Coleoptera: Curculionidae) (Barlow & Goldson, 1993) or *Microctonus hyperodae* Loan (Hymenoptera: Braconidae), might control the clover root weevil (Barratt *et al.*, 1996). However, when *M. aethiopoides* (Moroccan strain) and *M. hyperodae* were laboratory tested against *S. lepidus*, neither parasitoid showed good level of parasitism (Barratt *et al*., 1997) and researchers had to look for potential biological control agents abroad (Phillips *et al*., 2000; Nelson *et al*., 2004). Chlorpyrifos insecticide (ie: Lorsban™ 750 WG, Dow AgroSciences) is registered for the control of clover root weevil but is only advised to be used when sowing new pasture and was not considered a long term option to control this pest as it was not an option economically or environmentally sustainable (Willoughby *et al*., 1998) and researchers focused on biological control options.

After few years of research in Europe, New Zealand scientists found that an Irish strain of *M. aethiopoides* was a good candidate for the management of *S. lepidus* and this strain was released in 2006 in the North Island (Gerard *et al*., 2011). Since its release, the Irish wasp showed good parasitism levels of the adult clover root weevil and has established in New Zealand (Gerard *et al*., 2011; Phillips *et al*., 2010). However, the Irish *M. aethiopoides* is a parasitoid of the adult clover root weevil only and there was a need for a biological control agent for the most damaging larval stage in the soil.

During the search for biocontrol agents against *S. lepidus*, a strain of the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales), the strain F418, was isolated from cadavers of *S. lepidus* collected in Europe and was found to be highly virulent against the clover root weevil in laboratory conditions (Nelson *et al.*, 2004). Field trials established in 2005 showed a high variation in *B. bassiana* F418 population levels post application between the different sites, time and treatments tested; this highlighted the necessity of understanding the impact of abiotic and biotic factors on *B. bassiana* F418 population dynamics in soil to improve this biopesticide (Brownbridge *et al*., 2006).

1.2 Biocontrol of pest arthropods with entomopathogenic fungi

Insect pathology studies began with the work of Agostino Bassi who stated in 1835 that he could artificially transmit white muscardine fungus, since then named *Beauveria bassiana*, to silkworms, *Bombyx mori* L. (Lepidoptera: Bombycidae) and other species at will (Lord, 2005; Goettel *et al.*, 2010). Elie Metchnikoff (Ilya Mechnikov) discovered the green muscardine fungus, since then named *Metarhizium anispoliae* (Metschn.) Sorokin (Ascomycota: Hypocreales), while looking for a control method for the cereal cockchafer, *Anisoplia austriaca* Herbst (Coleoptera: Scarabaeidae) in 1878 (Zimmermann *et al*., 1995). Metchnikoff then found a process to mass produce *M. anispoliae* conidia on beer mash and his protégé Isaac Krassilstschik established the first field trials using mass produced entomopathogenic fungi around 1890 (Lord, 2005). However, research into entomopathogenic fungi for the biocontrol of arthropods reduced with the successful development and use of chemical insecticides and the interest for biocontrol agents only reappeared in the late 1950s (Goettel *et al.*, 2010), following growing concerns about the effects of synthetic chemical pesticides on human and environmental health. Since then, a large amount of research has been done and permitted the commercial development of numerous fungal biopesticides with *B. bassiana* and *M. anisopliae* representing nearly 70 %

of all actively commercialised mycoinsecticides and mycoacaricides (Faria *et al*., 2007). Failures of biopesticide applications are also very numerous and the reasons for their lack of efficacy are not well understood due to a lack of understanding of the ecology of fungus in field conditions (Bidochka, 2001). Significant progress has been made in biopesticide production but this has been mainly in mass production, stabilization and formulation of the fungi (Wraight *et al.*, 2001). Essential questions remain about fungal ecology in the soil and these answers are critical to underpin the use of biopesticides and facilitate their widespread adoption in agriculture.

1.3 Mode of action of entomopathogenic fungi

The infection process starts with the adhesion of the conidia to the arthropod cuticle. The attachment is due to non-specific hydrophobic interaction between the outer layer of the spore wall and the epicuticle of the arthropod (Boucias *et al.,* 1988). Subsequent formation of the germ-tube (Fig. 1.1) is determined by different factors such as the nutrient level on the host cuticle (St. Leger *et al*., 1992), susceptibility of the host, humidity and substances found on the arthropod cuticle such as peptides important for fungal recognition (Zimmermann, 2007). The germ-tube forms an appressorium and a penetration peg (Fig. 1.1) which penetrates the arthropod cuticle through a combination of mechanical forces and enzymatic digestion by enzymes such as chitinases, lipases and proteases (Fang *et al*., 2005; Wang & St. Leger, 2007; Zimmermann, 2007). Once the fungus successfully penetrates the cuticle, it forms yeast-like hyphal bodies called blastospores and invades the whole arthropod via the haemolymph (Zimmermann, 2007) (Fig. 1.1). During the infection process, some toxins are produced by *B. bassiana* such as bassianin, beauvericin, bassianolide, tenellin and oosporein (Vey *et al.,* 2001), which have different roles in the infection process such as enzyme disruption and membrane-damage (Vey *et al.,* 2001). The arthropod will finally succumb to mycosis and the blastospores in the haemolymph will form filamentous hyphae and invade the remaining tissue. Hyphae will emerge from the cadaver and produce spores on the surface of the arthropod (Boucias & Pendland, 1998 cited by Tartar & Boucias, 2004).

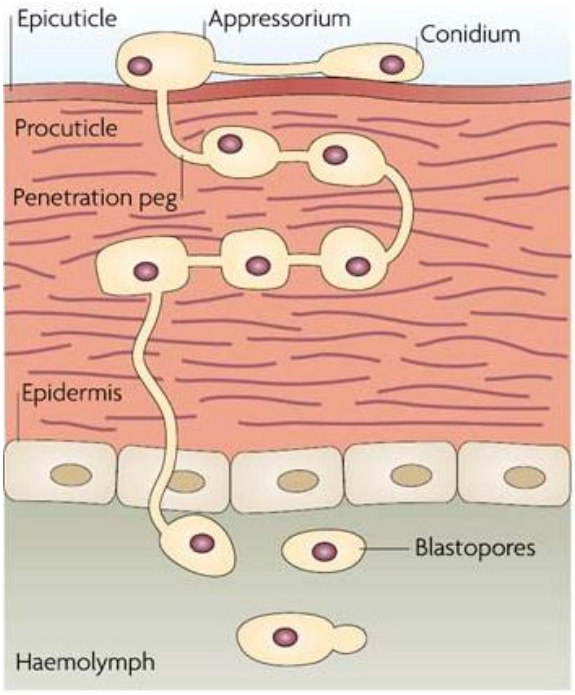

Figure 1.1 Infection by fungal entomopathogens. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Thomas & Read, 2007), copyright 2007.

1.4 Factors affecting the use of entomopathogenic fungi against soil dwelling insect pests

The success of an entomopathogenic fungus for the control of a soil-dwelling pest is dependent on the persistence and infectivity of the fungus in soil. Soil has been considered as a favourable environment for the use of entomopathogenic fungus in insect biocontrol, due to the protection from solar UV, the temperature buffer and the relative humidity usually high (Krueger & Roberts, 1997). However, soil is a highly complex environment with intricate interactions between microorganisms, plants, soil substrate and environmental factors. Numerous abiotic and biotic factors in soil can affect the persistence and virulence of entomopathogenic fungi (Jaronski, 2007). Yet, literature on the effect of environmental factors on entomopathogenic fungi is limited compared to research on plant pathogenic fungi and their microbial control agents and soil microbial populations (Jaronski, 2007).

1.5 Beauveria bassiana

Beauveria bassiana (Balsamo-Crivelli) Vuillemin (Ascomycota: Hypocreales: Cordycipitaceae) is an anamorphic specie of entomopathogenic fungi. *Beauveria bassiana* colonies on full-strength Sabouraud's dextrose and potato dextrose agar are characterised by cottony or woolly appearance of white colour becoming yellowish or rarely pinkish, and the reverse of the culture is colourless or yellow or more rarely pinkish (Rehner *et al*., 2011). *Beauveria bassiana* produces vegetative hyphae 1-2 µm wide, on which conidiogenous cells are produced in dense clusters (Rehner *et al*., 2011). The conidiogenous cells are flask-shaped with long rachis forming a zig-zag bearing the asexual spores called conidia (Fig. 1.2) (Brady, 1979; Inglis *et al*., 2001). Conidia are globose to broadly ellipsoidal and 2-3 µm × 2-3 µm (Rehner *et al*., 2011). *Beauveria bassiana* reproduces asexually, however, the teleomorph *Cordyceps bassiana* has been discovered in Asia (Li *et al*., 2001 cited by Meyling *et al*., 2009) and has been proved to be linked developmentally and phylogenetically to *B. bassiana* (Rehner *et al*., 2011).

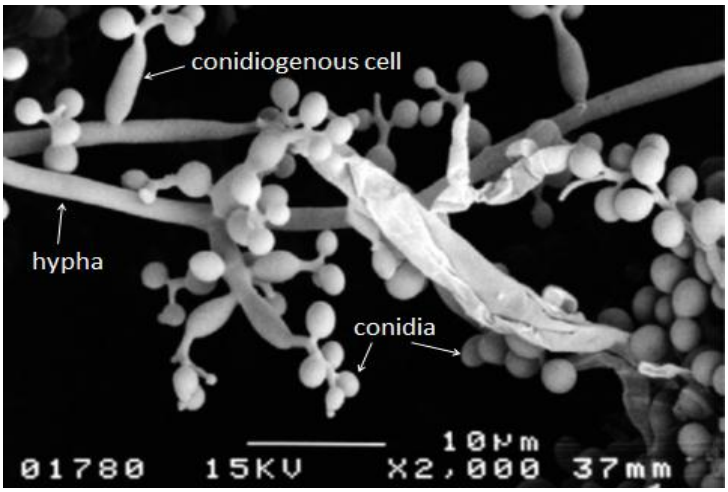

Figure 1.2 Scaning electron micrograph of *Beauveria bassiana* showing the structures (Glare, unpublished).

Beauveria bassiana is a soil-borne entomopathogenic fungus found worldwide in diverse habitats (Zimmerman, 2007) from soil, to air and even as endophytes in some plants (Wagner & Lewis, 2000; Posada & Vega, 2005; Quesada-Moraga *et al*., 2006). *Beauveria bassiana* is one of the most common fungus infecting insect (Boucias, 1998 cited by Tartar & Boucias, 2004) and has a very wide host range, infecting at least 700 species of insect belonging to 15 different orders (Li, 1988 cited by Zimmermann, 2007). Despite this host

diversity, each isolate of *B. bassiana* are usually restricted to a small host range (Zimmermann, 2007). *Beauveria bassiana* F418 has been tested against a range of invertebrates and showed no pathogenicity against bees, *Apis mellifera* L. (Hymenoptera: Apidae), earthworms *Aporrectodea caliginosa* Savigny, *Lumbricus rubellus* Hoff., *Lumbricus terrestris* L. (Haplotaxida: Lumbricidae), Argentine stem weevil *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae), Argentine stem weevil parasitoid *Microctonus hyperodae*, grass grub *Costelytra zealandica* White (Coleoptera: Scarabaeidae) and black beetle *Heteronychus arator* F. (Coleoptera: Scarabaeidae) (Nelson *et al*., unpublished). Whereas *B. bassiana* F418 showed pathogenicity against *S. lepidus* (Nelson *et al.*, 2004), alfalfa root weevil *Sitona discoideus*, painted apple moth *Orgyia anartoides* Walker (Lepidoptera: Lymantriidae) (Nelson *et al*., unpublished), Fuller's rose weevil, *Naupactus cervinus* Boheman (Curculionidae: Entiminae) (Mander, 2006) and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae).

1.6 Factors affecting *Beauveria bassiana* persistence and virulence in soil

1.6.1 Abiotic factors

Soil moisture

Persistence of *B. bassiana* in sterile or non-sterile soil generally decreases with increasing soil moisture (Lingg & Donaldson, 1981; Studdert *et al.*, 1990; Quintela *et al*., 1992). Lingg & Donaldson (1981) studied the survival of *B. bassiana* strain M-4 conidia at 10 °C in sterile soil (autoclaved) and non-sterile soil held at 25, 50 and 75 % saturation during 265 d and found that conidia survival decreased with the increasing soil moisture in both sterile and non-sterile soil. Conidia survival (half-time) in sterile soil was 85.1 d at 25 % saturation while 24.0 d at 75 % saturation and in non-sterile soil was 225.0 d at 25 % saturation while 33.3 d at 75 % saturation. Similarly, Quintela *et al*. (1992) studied the colony forming units (CFU) recovery of *B. bassiana* strain CP5 in autoclaved and non-autoclaved soil over 80 d at 17 °C and found that CFU recoveries were significantly higher in autoclaved and non-autoclaved soil at 25 % saturation compared to 75 % saturation. Studdert *et al*. (1990) studied *B. bassiana* strain ABG-6178 conidia survival at 16 °C in non-sterile soil (silt loam and peat)

held between 0 and -1500 bar during 50 weeks and found that conidia survival increased with decreasing soil moisture until permanent wilting point of -15 bar. Conidia survival (half-time) was 36.3 weeks at -15 bar in silt loam soil while 0.6 weeks at 0 bar (saturation) and was 15.2 weeks at -15 bar in peat soil while 0.5 weeks at 0 bar (saturation).

Clerk & Madelin (1965) studied the longevity of *B. bassiana* conidia after storage at 8, 18 and 25 °C at different relative humidity (RH) ranging from 0 to 75 % and found that conidia longevity decreased with increasing RH Similarly, Sandhu *et al*. (1993) found that *B. bassiana* conidia survival was better in storage at lower RH (0 to 53 % RH) compared to higher RH (75 to 98 %). Therefore, *B. bassiana* persistence in soil or in storage seems to be negatively correlated to moisture level. However, since the soil moisture is generally over 98 % RH even at the permanent wilting point (pF -4.2 or -15 bar) (Griffin, 1963), it is difficult to link studies in soil where the RH is always over 98 % RH with shelf life studies using lower RH.

Lingg & Donaldson (1981) discussed the possibility that the decrease of conidia with increasing soil moisture was due to either the direct impact of soil moisture on the physiology of the conidia or being due to an increase in CO_2 and decrease in O_2 in wetter soil affecting the fungus outgrowth. However, they did not test either.

Quintela *et al*. (1992) suggested that the decrease in *B. bassiana* CFU recovered in autoclaved and non-autoclaved soil at 75 % saturation compared to 25 % saturation was due to the presence of other microorganisms competing or inhibiting *B. bassiana* in wetter soil. They studied the recovery of bacteria, actinomycetes and fungi from autoclaved and non-autoclaved soil in presence of absence of *B. bassiana* but they did not study the impact of soil moisture on other microorganisms. Similarly, Studdert *et al*. (1990) discussed their finding of *B. bassiana* conidia survival being negatively affected by increasing soil moisture as being due to either an increase in bacterial activity in wetter soil which could be antagonistic to *B. bassiana* conidia or due to physiological causes of the soil moisture level itself. Quintela *et al*. (1992) and Studdert *et al*. (1990) suggested that *B. bassiana* persistence in wet soil was affected by antagonistic microorganisms; however they did not investigate their hypothesis further.

Regarding the virulence against host arthropod, Luz & Fargues (1999) showed that *B. bassiana* INRA 297 required over 96 % RH for the infection of *Rhodnius prolixus* Stal (Hemiptera: Reduviidae). Since the relative humidity in soil is over 98 % RH even at the permanent wilting point, the soil moisture might not be an important factor on the virulence of *B. bassiana* against host arthropod.

Soil temperature

Beauveria bassiana persistence in soil decreases with increasing temperature (Lingg & Donaldson, 1981; Studdert *et al.*, 1990; Quintela *et al*., 1992). Lingg & Donaldson (1981) studied the survival of *B. bassiana* M-4 and CLB-2 strains in sterile and non-sterile soil at -15, 10, 25 and 55 °C during 265 d and found that *B. bassiana* survival (half time) decreased with increasing temperature and no *B. bassiana* could be recovered after 10 d in both soils at 55 °C. Similarly, Quintela *et al*. (1992) found a decrease in *B. bassiana* strain CP5 CFU with increasing temperature in autoclaved and non-autoclaved soil after 80 d at 17, 24 and 30 °C. Studdert *et al*. (1990) also observed that *B. bassiana* strain ABG-6178 conidia survival decreased with increasing temperature in different experiments between 10 and 50 °C in two types of non-sterile soil.

The impact of soil temperature on *B. bassiana* persistence seems to parallel the impact of temperature on *B. bassiana* shelf life. Clerk & Madelin (1965) observed that *B. bassiana* conidia longevity decreased with increasing temperature when stored at 8, 18 and 25 °C. Sandhu *et al.* (1993) also observed that *B. bassiana* conidia survival was better at low temperature ranging 0 to 20 °C compared to higher temperature ranging from 30 to 40 °C.

Following their finding on the effect of soil temperature on *B. bassiana* survival, Studdert *et al*. (1990), suggested that it could be due to the direct effect of increasing temperature such as observed in shelf life studies but also could be due to the indirect effect of other microorganisms which are more active at higher temperatures and could be responsible for *B. bassiana* conidia degradation.

Beauveria bassiana virulence against host arthropod is influenced by temperatures (Studdert & Kaya, 1990; Ekesi *et al*., 1999; Krueger *et al*., 1991). Studdert & Kaya (1990) observed that *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) mortality after 10 d of incubation in a fine sandy loam soil inoculated with 1×10^8 *B. bassiana* strain ABG-6178 conidia/cm^3 of soil was null at 8 °C, 69 % at 16 °C and 90 % at 24 °C. Similarly, Krueger *et al*. (1991) observed that the incubation time to obtain mortality of *Blissus leucopterus leucopterus* Say (Hemiptera: Lygaeidae) in soil inoculated with *B. bassiana* increased with decreasing temperature from 30 to 20 °C. These virulence results in soil are similar to results in laboratory experiments without soil. Ekesi *et al.* (1999) observed that the percentage mortality of *Megalurothrips sjostedti* Trybom (Thysanoptera: Thripidae) by *B. bassiana* strains ICIPE 53 increased from 27.4 % at 15 °C to 88.4 % at 20 °C and to 100 % at 25 °C. The effect of temperature on *B. bassiana* virulence in soil correlates to the increase in physiological activity of *B. bassiana* function of temperature (Ekesi *et al*., 1999). However,

when screening the virulence of potential *B. bassiana* isolates for biological control of insect pests, attention must be taken to study the virulence at the temperatures occuring in the environment in which the isolate will be used (Doberski, 1981a and 1981b). Also, when screening fungal isolates for biological control of insect pests, the isolates chosen are usually from a similar climatic region (Doberski, 1981; Ekesi *et al*., 1999) as fungus thermoactivity is linked to their climatic region of origin. Fernandes *et al*. (2008) found that *Beauveria bassiana* isolates from cold climatic regions were more cold-active than isolates from regions closer to the equator. Ekesi *et al*. (1999) found that the isolate with the best potential for the control of legume flower thrips, *M. sjostedti*, in Kenya was an isolate of *M. anisopliae* from Kinshasa, Democratic Republic of the Congo.

Effect of organic matter content

The soil organic content can affect *B. bassiana* and other entomopathogenic fungi persistence and virulence in soil (Studdert *et al*., 1990; Studdert & Kaya, 1990; Vänninen *et al*., 2000). Studdert *et al*. (1990) found that *B. bassiana* conidia strain ABG-6178 survival was better in fine sandy loam soil containing less than 1 % organic matter than in peaty muck soil containing 62 % organic matter and they observed the same results in the different experiments done in laboratory conditions. In one of their experiments, *B. bassiana* conidia half-life was 36.3 weeks in fine sandy loam soil at -15 bar and 16 °C while the survival was 15.2 weeks in the peaty muck soil. Vänninen *et al*. (2000) observed that *M. anisopliae* strain SF85-60 persistence was better in a clay soil than in a peat soil *in situ*, 1, 2 and 3 years following the inoculation of field soil with *M. anisopliae* conidia. Studdert *et al*. (1990) and Vänninen *et al*. (2000) concluded that the lack of persistence in soil with high organic matter content was likely due to the higher microbial activity in soil rich in organic matter leading to antagonism against entomopathogenic fungi such as the inhibiting action of *Penicillium urticae* Bainier (Ascomycota: Eurotiales) against *B. bassiana* (Lingg & Donaldson, 1981)

Studdert & Kaya (1990) observed that *S. exigua* mortality due to *B. bassiana* was lower in a peaty muck soil than in a fine sandy loam soil and concluded that it was probably due to antagonism by other microorganisms more present in the high organic soil compared to the low organic soil.

The organic matter content of a soil can change the C/N ratio which can influence the fungus growth and virulence. Smith & Grula (1981) showed that a carbon source is needed for *B*. *bassiana* germination and a nitrogen source is needed for continuous growth otherwise

lysis occurs. James (2001) found that a nitrogen source is also necessary for *B*. *bassiana* germination. Shah *et al.* (2005) showed that *M. anisopliae* radial growth and virulence against *T. molitor* were influenced by the C/N ratio of the media on which *M. anisopliae* grew.

Effect of fertilisers

The persistence of *B. bassiana* in soil can be affected by the addition of fertilisers; however the different studies done gave inconsistent results (Lingg & Donaldson, 1981; Rosin *et al*., 1996; Townsend *et al*., 2003). Lingg & Donaldson (1981) studied *B. bassiana* strain M-4 conidia survival in sterile and non-sterile soil with addition of different nitrogen sources and found that the addition of urea (CH_4N_2O) led to an increase of *B. bassiana* persistence in sterile soil with 2,545 % of *B. bassiana* recovered after 22 d in sterile soil with urea compared to 88 % *B. bassiana* recovered in control sterile soil. The addition of urea to non-sterile soil led to no recovery of *B. bassiana* after 22 d and 50 % recovery in the control non-sterile soil. Rosin *et al.* (1996) found no difference in *B. bassiana* CFU recovery in sterile soil with 0, 280 or 560 kg/ha of urea after 7 d incubation. Similarly, Townsend *et al*. (2003) found no difference in *B. bassiana* B17 (now called F418) CFU recovery after 4 weeks in non-sterile soil amended with different nitrogen and phosphate fertilisers or no fertiliser.

Lingg & Donaldson (1981) concluded that the addition of urea probably stimulated the microorganisms present in non-sterile soil leading to antagonistic activity against *B. bassiana*. In more general studies of microbial populations in soil with different nitrogen additions, high nitrogen addition seems to be detrimental to fungal populations (Bittman *et al*., 2005; de Vries *et al*., 2006). Bittman *et al*. (2005) found that the addition of ammonium nitrate at high rate (100 kg NH_4-N/ha) led to a decrease of fungal biomass (in meter of fungal hyphae/g soil) in field soil compared to control soil with no addition of fertiliser. De Vries *et al*. (2006) observed that the fungal/bacterial ratio decreased in a grass-clover field with increasing nitrogen application (slurry and manure) from 0 to 120 kg N/ha and found that bacterial activity increased with increasing nitrogen application. Sarathchandra *et al.* (2001) studied the impact of long term application of urea and superphosphate onto soil microbial population in pasture soil and found that *Beauveria brongniartii* (Sacc.) Petch (Ascomycota: Hypocreales) population decreased with increasing urea application from 0.8 % of the total fungal population without urea application to 0 % with 400 kg N/ha/year; however this result could not be analysed statistically.

Other abiotic factors

Soil texture and soil pH can have an effect on entomopathogenic fungi, however, their effects have not been studied deeply and results are sometimes contradictory. Rath *et al.* (1992 cited in Jaronski, 2007) found that the frequency of *M. anisopliae* isolated from Tasmanian pastures was correlated to the soil texture but not to soil pH, with more isolations made from loam and clay loam soils than from sandy loam and clay soils. Harrison & Gardner (1991 cited in Jaronski, 2007) found no correlation between *B. bassiana* isolation and soil types in 19 pecan orchards in Georgia. Studdert *et al.* (1990) found that *B. bassiana* survival was better in a fine sandy loam soil than in a peaty muck soil. Studdert & Kaya (1990) observed a difference in virulence of *B. bassiana* against *S. exigua* in fine sandy loam and peaty muck soil. Groden & Lockwood (1991) studied the fungistasis effect of soils from Rhode Island and Michigan on *B. bassiana* and found that fungistasis increased with increasing pH. Rousk *et al*. (2010) showed that fungal communities were not strongly affected by differences in soil pH compared to bacterial communities.

1.6.2 Biotic factors

Soil microorganisms

Soil microorganisms play an important role in persistence of entomopathogenic in soil. Huber (1958 cited in Clerk, 1969) reported the absence of germination of *B. bassiana* conidia in fresh garden soil and the uplifting of this inhibition once the soil was sterilised. Lockwood (1964) reviewed previous work on soil fungistasis and concluded that fungistasis was described as fungal spores being unable to germinate in natural soil due to the inhibiting action of other soil microorganisms by competition for nutrients or production of antibiotics and that inhibition could be removed by soil sterilisation or addition of nutrients. Clerk (1969) reported the inhibition of *B. bassiana* and *Isaria farinosa* (Holmsk.) Fr. (Hypocreales: Cordycipitaceae) (formerly *Paecilomyces farinosus*) germination in natural soil extracts and observed that the inhibition could be reduced when the soil extracts were filtered or if water extract of silk from *Bombyx mori* cocoon (which is a host of *B. bassiana*) was added to the soil extract. Clerk (1969) concluded that fungistasis present in natural soil extract was due to bacteria and probably other microorganisms and that the presence of host arthropod could counteract the fungistasis. Similarly, Walstad *et al*. (1970) observed that *B. bassiana* and *M. anisopliae* could not germinate in non-sterile soil or leaf litter and the inhibition could be

removed when the substrate was sterilised and concluded that the inhibition was due to fungistasis by other microorganisms. They also noted that both fungi could germinate on host *Hylobius pales* Herbst (Coleoptera: Curculionidae) even when the body wall of the weevil was not sterile. More recently, Shimazu *et al*. (2002) observed the absence of germination of *B. bassiana* conidia in non-sterile soil while germination occurred in sterile soil when studying the density dynamics of *B. bassiana* introduced into forest soil. Shimazu *et al*. (2002) also followed the density of bacteria, actinomycetes and fungi in the forest soil before and after inoculation of the soil with *B. bassiana* to investigate the potential influence of *B. bassiana* on the density of other soil microorganisms. They found that the density of bacteria and actinomycetes did not change after addition of *B. bassiana* to the soil. The increased density of fungi noted following the addition of *B. bassiana* was directly attributed the addition of *B. bassiana*. They concluded that the lack of any effect from the addition of *B. bassiana* to forest soil was probably the result of the inert state of *B. bassiana* conidia in non-sterile soil. They also observed a gradual decrease in *B. bassiana* densities down to $1/10^{th}$ the initial density over 12 months.

However, none of these papers (Huber, 1958; Clerk, 1969; Walstad, 1970) could identify a specific microorganism being antagonist to *B. bassiana*. Lingg & Donaldson (1981) reported *P. urticae* as an inhibitor of *B. bassiana* after adding *P. urticae* filtrate to sterile soil inoculated with *B. bassiana* and being unable to recover any *B. bassiana* after 12 d in these conditions whereas a 10 fold increase of *B. bassiana* was observed in sterile soil without *P. urticae* filtrate.

Lingg & Donaldson (1981) also added some carbon and nitrogen sources to non-sterile soil inoculated with *B. bassiana* to confirm the observations made by Clerk (1969) and Lockwood (1964) that fungistasis effects in non-sterile soil could be overcome by addition of nutrients. However they found the opposite with a more pronounced inhibition in non-sterile soil with added nutrients showing that fungistasis was not due to a competition for nutrients but due to other microorganisms being antagonistic to *B. bassiana*.

Sharapov & Kalvish (1984) found that fungistasis levels varied between soils and increased in soils with higher organic matter contents. Similarly, Groden & Lockwood (1991) studied the level of fungistasis of different soils on *B. bassiana* germination and the effect on *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) mortality and they observed that fungistasis levels varied between soils and years and that fungistasis was stronger in soil with neutral pH compared to more acidic soil probably due to bacteria and actinomycetes preference to less acidic soils. They also found that *B. bassiana* was less virulent (increase in LD_{50}) in soils with more fungistasis. Therefore, soil microorganisms can

inhibit *B. bassiana* germination and growth in soil; however a few studies have reported the ability of some *B. bassiana* strains to germinate and conidiate in non-sterile soil (Studdert *et al.,* 1990; Quintela *et al*., 1992). Studdert *et al.* (1990) observed a 200 % increase in CFU recovery of *B. bassiana* strain IL-116 in non-sterile soil over the first two weeks after inoculation in the absence of host arthropod and concluded that *B. bassiana* strain IL-116 could conidiate in non-sterile soil. Similarly, Quintela *et al.* (1992) observed a 200 % increase in CFU recovery of *B. bassiana* strain CP5 in non-sterile soil 52 d after inoculation in the absence of host arthropod and concluded that the increase was probably due to conidiation. Soil microorganisms can also degrade *B. bassiana* propagules (Fargues *et al*., 1983; Markova, 1991). Fargues *et al.* (1983) observed *B. bassiana* blastospores degraded by antagonistic bacteria and amoebae in soil. Markova (1991) observed *B. bassiana* parasitised by fungus *Melanospora parasitica* (now *Syspastospora parasitica* (Tul.) P.F. Cannon & D. Hawksw. (Ascomycota: Hypocreales). Posoda *et al*. (2004) also observed *S. parasitica* infecting *B. bassiana*. Krauss *et al*. (2004) studied the interactions between some entomopathogenic fungi and some mycoparasites and found that *B. bassiana* was attacked by mycoparasite *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams (Ascomycota: Hypocreales).

Effect of non-host arthropods

Entomopathogenic fungi viability could be affected by the presence of non-host arthropods via non-specific attachment and germination on the arthropods cuticle or digestion by non-host arthropods (Boucias *et al*., 1988; Sitch & Jackson, 1997; Hajek & Eastburn, 2003; Lacey *et al*., 1988; Dromph, 2001; Ment *et al*., 2012). Non-host arthropod will be defined in the present thesis as an arthropod not susceptible at a specific inoculum level to a specific entomopathogenic fungus. Boucias *et al.* (1988) observed that *B. bassiana* and other entomopathogenic fungi could attach to non-host cuticle due to binding forces. They used ghost cuticle, which means that the skeletal structure was used intact but the internal tissues were eliminated (Smith *et al*., 1981), therefore the attachment of *B. bassiana* to non-host arthropods was in absence of the arthropod immune system.

Sitch & Jackson (1997) observed that *Lecanicillium lecanii* (Zimm.) Zare & W. Gams (Ascomycota: Ascomycetes) (formerly *Verticillium lecanii*) could attach and germinate onto the cuticle of non-host coleopteran *Bembidion obtusum* Audinet-Serville (Coleoptera: Carabidae). Similarly, Hajek & Eastburn (2003) studied the attachment and germination of *Entomophaga maimaiga* Humber, Shimazu & R.S. Soper (Zygomycota: Entomophthorales) onto host and non-host arthropods cuticle and found that *E. maimaiga* could attach onto non-host arthropods cuticle and a small percentage could even germinate onto non-host

arthropods cuticle. Ment *et al.* (2012) studied the interaction between *gfp* transformed *Metarhizium* spp. and the non-host tick *Hyalomma excavatum* Koch (Acari: Ixodidae) and found that *gfp* transformed *Metarhizium* spp. could geminate and form appressoria on cuticle of non-host tick but could not penetrate the cuticle and died after a few days. This non-specific attachment and germination onto non-host arthropods cuticle is detrimental to entomopathogenic fungi persistence and virulence in soil since the attachment to non-host arthropods could prevent interaction with the host arthropod and the germination of conidia on non-host arthropods may lead to their loss.

Another effect could be inactiviation after ingestion. Dromph (2001) reported the loss of conidial viability of *M. anisopliae* after digestion by collembolans with 24.3 and 54 % germination of *M. anisopliae* after passage through the guts of *Proisotoma minuta* Tullberg (Collembola: Isotomidae) and *Folsomia fimetaria* L. (Collembola: Isotomidae) respectively compared to 98.8 % germination for the control without passage through arthropod guts. Lacey *et al*. (1988) observed partially digested conidia of *M. anisopliae* inside the guts of host insect *Culex quinquefasciatus* Say (Diptera: Culicidae). Non-host arthropods can potentially decrease the persistence of entomopathogenic fungi in soil via the passage through their digestive system.

Non-host arthropods can also have a negative impact onto entomopathogenic fungi virulence by preventing the fungi coming into contact with potential host arthropods due to the retention time inside the non-host arthropods gut. Schabel (1976) found conidia of *M. anisopliae* inside the guts of *H. pales* up to 5 d after ingestion. However, the ingestion of entomopathogenic fungus by non-host arthropods can also have some positive impacts such as the dispersion to soil zones where the fungus was not present prior and could therefore lead to an increase of contact with host arthropods as demonstrated by Dromph (2003).

Host arthropods

The presence of host arthropods is a major factor for entomopathogenic fungus dynamics in soil. Walstad *et al*. (1970) concluded that *B. bassiana* was restricted to the host arthropods for their development as *B. bassiana* did not grow saprophytically in non-sterile soil. Similarly, Kessler *et al*. (2004) observed that *B. brongniartii* persistence decreased by 88 % in absence of host insect *Melolontha melolontha* L. (Coleoptera: Scarabaeinae) 16 month after application of *B. brongniartii* to meadows compared to a decline of 27 % on average for sites with host *M. melolontha*.

Plant

The presence of plants in the close vicinity of *B. bassiana* and other entomopathogenic fungi can affect their persistence in soil (Hu & St. Leger, 2002; Bruck, 2005). Hu & St. Leger (2002) studied the fate of *M. anisopliae* in the bulk soil and the rhizophere of a cabbage (*Brassica oleracea*, L., Brassicales: Brassicaceae) field. The rhizosphere includes the few millimetres of soil surrounding the plant roots and is the centre of numerous interactions between roots, root exudates, microorganisms and invertebrates (Bais *et al*., 2006). Root exudates are mainly composed of low molecular weight compounds (i.e. amino acids, organics acids, sugars, phenolics) and other secondary metabolites which regulate the surrounding microbial community (Walker *et al*., 2003). Hu & St. Leger (2002) found that *gfp* transformant *M. anisopliae* ARSEF 1080 GMa populations were maintained at 10^5 propagules/g soil in the rhizosphere of cabbage plant in a field experiment whereas fungal population in bulk soil declined from 10^5 to 10^3 propagules/g soil several months after application. They suggested that the proximity of roots and root exudates was responsible for the effect observed. They described for the first time a rhizosphere competent entomopathogenic fungus. Schmidt (1979) describes rhizosphere competence as the enhanced growth of microorganisms in the immediate vicinity of the developing roots. Following Hu & St. Leger (2002) finding, Bruck (2005) showed that *M. anisopliae* populations (strain F52) increased in the rhizosphere soil of *Picea abies* (L.) Karst. (Pinales: Pinaceae) and was significantly greater than in the bulk part of soil (peat and bark-base potting mix) after 29 weeks. McKinnon (2011) found that populations of *B. bassiana* and *B. caledonica* Bissett & Widden (Ascomycota: Hypocreales) isolates were greater in the rhizosphere soil of *Pinus radiata* D. Don (Pinales: Pinaceae) compared to the bulk soil in non-sterile soil.

Beauveria bassiana can also have a stronger relationship with the plant by becoming an endophyte in a wide range of plant species. Bing & Lewis (1991) found that *B. bassiana* applied to the foliage could colonise the corn plant (*Zea mays*, L., Poales, Poaceae), move within it and could persist during the season providing suppression of the European corn borer *Ostrinia nubilalis* Hubner (Lepidoptera: Crambidae). Since then, a number of important publications have reported *B. bassiana* as an endophyte in plant species. Posada & Vega (2005) successfully inoculated cocoa seedlings (*Theobroma cacao*, L., Malvales, Malvaceae) *in vitro* with *B. bassiana* (IC-5486 and CS16-1 strains) conidia suspensions. *Beauveria bassiana* was recovered from roots, stems and leaves two month after inoculation and was even found as an epiphyte on the cocoa stems. Posada & Vega (2006) had similar findings with coffee seedlings (*Coffea arabica*, L., Gentianales, Rubiaceae). Gomez-Vidal *et al*. (2006) inoculated wounded date palm (*Phoenix dactylifera*, L., Arecales, Arecaceae)

petioles *in vitro* and *in vivo* with *B. bassiana* (119 and 33 strains) and the fungus could be recovered 30 d later up to 3 cm from the inoculation point. Quesada-Moraga *et al.* (2006) sprayed *B. bassiana* (EABb 04/01-Tip strain) conidia onto opium poppy (*Papaver somniferum*, L., Ranunculales, Papaveraceae) foliage and all plants treated were colonised by the fungus between 24 and 144 h after treatment and leaves formed after treatment were also colonized. Akello *et al.* (2007) inoculated banana (*Musa spp.*, L., Zingiberales, Musaceae) roots and rhizome with *B. bassiana* (G41, S204 and WA strains) using three different methods (roots and rhizome dipped in conidial suspension, conidial suspension injected into the plant rhizome and growing the plants in sterile soil mixed with *B. bassiana* colonized rice). *Beauveria bassiana* colonised banana plant tissue using all three methods although the root and rhizome dipping method gave a better colonization. Tefera & Vidal (2009) inoculated sorghum (*Sorghum sp.*, L., Poales, Poaceae) with *B. bassiana* (Bb-04 strain). They used different inoculation methods. They inoculated the seeds (by immersion in conidia suspension), the leaves (conidia suspension sprayed onto the leaf), and the soil (conidia suspension applied around the root zone of each seedling). They also used different plant growth medium (sterile soil, non-sterile soil and vermiculite). They found that *B. bassiana* could establish as an endophyte in roots, stems and leaves using the different inoculation method but colonization depended on the plant growth medium used (i.e. stem and leaf were not colonized by *B. bassiana* after seed inoculation method when plants were growing in non-sterile soil. Gurulingappa *et al.* (2010) inoculated cotton (*Gossypium hirsutum*, L., Malvales, Malvaceae), wheat (*Triticum aestivum*, L., Poales, Poaceae), bean (*Phaseolus vulgaris*, L., Fabales, Fabaceae), tomato (*Lycopersicum esculentum*, L., Solanales, Solanaceae) and pumpkin (*Cucurbita spp.*, Juss., Cucurbitales, Cucurbitaceae) plants with *B. bassiana* by spraying the leaves with a conidial suspension. The plants were subsequently successfully colonized by *B. bassiana*. They also found that if aphids *Aphis gossypii* Glover (Hemiptera: Aphididae) were feeding on cotton leaves colonised by *B. bassiana*, their reproduction was slowed. There are numerous reports of successful colonisation of plants by *B. bassiana* using different inoculation methods (seed dipping, foliar spray, stem injection, soil inoculation).

There are several reports of *B. bassiana* colonised plants reducing damage by insect pests (Akello *et al.*, 2008; Bing & Lewis, 1991) which are attributed to the toxic metabolites produced by *B. bassiana*. Akello *et al.* (2008) demonstrated that banana plants colonised by *B. bassiana* could reduce the population of the banana weevil *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae) and therefore the plant damage caused by this pest. Colonisation of plants by endophytic *B. bassiana* could provide persistence of the biological agent *in situ* and pest management.

1.6.3 Pre-application factors

Pre-application factors such as fungus formulation or media of growth can affect the persistence and virulence of the entomopathogens in soil (Fargues *et al*., 1983; Shah *et al*., 2005; Nelson *et al*., 2004). Fargues *et al*. (1983) studied the persistence of naked and clay-coated blastospores of *B. bassiana* pathotype No. 18 in soil at 20 °C and found that naked blastospores could not be recovered after 3 weeks incubation whereas clay-coated blastospores recovery was still high after two months of incubation, showing the importance of the formulation on entomopathogen persistence in soil. Similarly, Nelson *et al*. (2004) compared the persistence of *B. bassiana* F418 formulated on rice and on different biopolymer granules and found that the rice laden formulations had better persistence in soil than the granule formulations one year following the application in pasture.

Shah *et al*. (2005) studied the impact of different media of growth on the virulence of *M. anisopliae* V245 and found that KCl medium (8 % glucose, 2 % peptone, 5.5 % KCl), medium with low C/N ratio of 10:1 (0.6 % glucose, 1 % peptone), medium with high C/N ratio of 75:1 (9.1 % glucose, 1 % peptone), medium with 2 % peptone and medium with 1 % yeast extract produced conidia significantly more virulent than medium with intermediate C/N ratio of 35:1 (4 % glucose, 1 % peptone) and potato dextrose agar (PDA) medium.

1.7 Techniques to isolate, quantify and identify entomopathogenic fungi in soil

1.7.1 Isolation and quantification techniques

The standard '*Galleria* bait method' (Zimmermann, 1986) is used to isolate indirectly entomopathogenic fungi from soil samples by using was moth larvae *Galleria mellonella* L. (Lepidoptera: Pyralidae) as a susceptible host. This technique is used for the ecological study of the distribution of entomopathogenic fungi in the environment (Meyling & Eilenberg, 2006; Meyling *et al*., 2011; Meyling *et al*., 2012). The insect bait method can also be done using different insects such as *T. molitor* (Hugues *et al.*, 2004; Vänninen, 1996), *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) (Vänninen, 1996), *Acanthocinus aedilis* L. (Coleoptera: Lamiinae) (Vänninen, 1996) or *Coccus viridis* Green (Hemiptera: Coccidae)

(Jackson *et al*., 2012). The insect bait method is a simple indirect technique to isolate entomopathogenic fungi from the soil environment; however this method presents some drawbacks (Jaronski, 2007; Scheepmaker & Butt, 2010). This method can only detect fungal species/strains to which the bait insect is susceptible, therefore underestimating the real number of species/strains present in soil (Scheepmaker & Butt, 2010). Also, different fungi can be isolated depending on the temperature of incubation of the bait insect (Jaronski, 2007). Finally, this method is qualitative only; it cannot be used for the quantification of the fungus in soil (Kessler & Keller, 2003 cited in Jaronski, 2007).

The most common direct technique to isolate and estimate populations of entomopathogenic fungi such as *B. bassiana* in soil is by dilution plating onto a semi selective media (Davet & Rouxel, 2000; Paulitz, 2000). This technique consists of sampling a known amount of soil followed by serial dilution of the soil and plating of the dilutions onto a semi selective media adapted for a specific fungus. The plates are then incubated under specified conditions and the CFU are counted (Davet & Rouxel, 2000). The dry weight of the soil used for the dilution is recorded and using a mathematic formula, an estimation of the number of *B. bassiana* CFU per gram of dry soil can be calculated (Paulitz, 2000).

Semi selective media contains nutrients to promote the growth of the selected fungus and specific antibiotic and/or fungicide to inhibit the growth of unwanted non-target organisms (Paulitz, 2000). *Beauveria bassiana* and other *Beauveria* spp. can be isolated on potato dextrose agar (PDA) medium, Sabouraud dextrose agar (SDA) medium and media derived from SDA containing different levels of peptone and glucose (Chase *et al*., 1986; Strasser *et al.*, 1996; Kessler *et al*., 2003; Meyling & Eilenberg, 2006). These nutrient media are usually amended with variable amounts of tetracycline, streptomycin, cyclohexamide and Dodine (Chase *et al*., 1986; Strasser *et al.*, 1996; Kessler *et al*., 2003; Meyling & Eilenberg, 2006). Tetracycline and streptomycin are bactericide and cycloheximide is a fungicide.

The technique of dilution plating onto semi selective media is cost efficient and easy to use. However, there are several drawbacks with this technique as this method does not allow differentiation between introduced and native strains of the same fungus (Green & Jensen, 1995) which is an issue when monitoring the persistence of an introduced biological control agent (BCA) if native fungal strains are present.

Also, this technique does not differentiate between different propagule types such as mycelia and conidia (Knudsen & Bin, 1990) and, thus, might not correlate with the actual fungal biomass (Paulitz, 2000). Warcup (1955) found that most of the CFU developing on soil dilution plates originated from conidia with fungi such as *Trichoderma* sp. Pers. (Ascomycota: Hypocreales) and *Penicillium* sp. Link (Ascomycota: Eurotiales); showing that

either of these fungi were only present as dormant spores or that active mycelium could not be isolated using soil dilution plating on semi selective media. Hohmann (2010) used a filtration dilution plating method to estimate CFU from spores and from mycelial fragments of *Trichoderma hamatum* (Bonord.) Bainier (Ascomycota: Hypocreales) in soil. In his study, the total CFU numbers were estimated by normal dilution plating method, then the soil suspension was filtered through three to four layers of miracloth to retain the mycelial fragments and the filtered suspension was plated for the estimation of CFU arising from spores only. The CFU from mycelial fragments only were estimated by subtracting CFU from spores only from CFU from both spores and mycelial fragments. However, the length of mycelial fragments obtained after 10 min shaking of soil suspensions before the plating was not measured. Therefore, it was not known if the sizes were homogenate and the estimation of CFU from mycelial fragments could not be correlated to the real mycelial biomass as for example a long piece of mycelia would give rise to only 1 CFU whereas the same fragment length cut into 5 pieces would give rise to 5 CFU. It is also believed that the dilution plating technique cannot detect hyphal fragments because of the potentially strong binding of the hyphal fragments to soil particles, eliminating it during the serial dilutions (Jaronski, 2007).

A technique to estimate the fungal radial growth in soil was developed by Grose *et al*. (1984) and called the “soil-sandwich” technique. The “soil-sandwich” method consisted of a nucleopore filter, with a fungal inoculum in its centre, sandwiched between two layers of soil. After incubation, the filter was removed from the soil and the hyphal radial growth was measured. The fungus on the filter can be stained to facilitate the observation (Wakelin *et al*., 1999). This “soil-sandwich” method allows monitoring of active hyphal growth in soil only.

Immunological techniques can also be used to quantify fungus in soil (Paulitz, 2000). Thornton (2004) developed a monoclonal antibody specific for the active growth of the mycelium of *Trichoderma* sp. to monitor their saprophytic growth dynamics. However, the use of immunological technique to monitor dynamics of fungi has a high cost and the development of an antibody for a specific fungus is complicated (Paulitz, 2000).

The use of recent molecular techniques such as quantitative real time PCR (q-PCR) and quantitative real time reverse transcription PCR (qRT-PCR) allows the quantification of DNA or RNA from fungal structures in soil (Filion *et al*., 2003; Fang & Bidochka, 2006). These techniques are based on the use of specific DNA (or cDNA) probes labelled with fluorescent reporters, producing a fluorescent signal during the PCR amplification (Filion *et al*., 2003; Fang & Bidochka, 2006) and allow the detection and quantification of specific genes expressed during different stages of fungal development. Fang & Bidochka (2006) studied the expression of genes involved in germination, conidiogenesis and pathogenesis using

qRT-PCR. Pathan *et al*. (2007) analysed the gene expression patterns of *B. bassiana* when grown on insect cuticular extracts and synthetic media using qRT-PCR. However, these techniques involve considerable time and expenses to identify and optimise the developmental stage to determine specific probes. Also, the specific probes are unlikely to differentiate between an introduced and a native strain of the same fungal species.

The use of microscopy together with fungal stains or dye such as fluorescein isothiocyanate (FITC) can allow the monitoring of fungal growth in soil (Mander, 2006). Mander (2006) labelled conidia of *B. bassiana* with FITC and studied the conidial viability and germination of this fungus in soil using fluorescent microscopy. This technique can be improved by the use of marker such as β-glucuronidase gene (GUS) (Green & Jensen, 1995) or the green fluorescent protein (*gfp*) gene introduced in the genome of the fungus. Such *gfp* transformed fungi can be observed under epifluorescence microscopy. Orr & Knudsen (2004) used a *gfp* transformed strain of *Trichoderma harzianum* Rifai (Ascomycota: Hypocreales) and image analysis software to monitor the biomass of *T. harzianum* in non-sterile soil.

The *gfp* from the jellyfish *Aequorea victoria* Murbach & Shearer (Leptomedusae: Aequoreidae) was developed as a marker by Chalfie *et al.* (1994) and transformed into a fungus for the first time by Spellig *et al.* (1996). Since this first transformation, the use of the *gfp* reporter helped improving the understanding of fungal ecology (Lorang *et al*., 2001), as long as the marker does not change the fitness of the transformed organism (Paulitz, 2000). The addition of the selective marker hygromycin B phosphotransferase gene (*hph*) in transformed *gfp* fungus gives the fungus resistance to the antibiotic hygromycin B and allows the growth of these *gfp-hph* transformed fungi on selective media amended with hygromycin B; therefore, inhibiting the growth of other non-transformed fungi which improves the use of the soil dilution plating method. Hu & St. Leger (2002) used a *gfp* transformed *M. anisopliae* to study the ecology of this fungus in non-sterile soil. Such *gfp* transformation has been used with *B. bassiana* (Wu *et al*., 2008), however not for ecological studies in soil.

All these *in vivo* and *in vitro* techniques present both advantages and disadvantages. The *in vivo* techniques such as insect bait, dilution technique on selective media and soil sandwich method are easy and low cost techniques but these are semi quantitative at best (dilution on selective media) and cannot differentiate between propagule types, apart from the soil sandwich which can only estimate the mycelial component. The use of a *gfp* fungus can allow the visualisation and quantification of the population dynamics in soil; however the creation of a *gfp* fungus can be challenging and the visualisation technique used to follow the population dynamics by Orr & Knudsen (2004) is time consuming. The *in vitro* techniques such as molecular and immunological techniques can estimate the fungal

population and bring information about the propagules dynamics; however these techniques are costly and require lengthy optimisation.

1.7.2 Identification techniques

Identification of fungal species based on phenotypical characteristics only is not specific enough for the distinction of species within a genus such as *Beauveria* (Rehner & Buckley, 2005) since different species of *Beauveria* such as *B. australis*, *B. kipukae*, *B. pseudobassiana* and *B. varroae* are morphologically similar to *B. bassiana* (Rehner *et al*., 2011). The identification of *Beauveria* spp. and *B. bassiana* strains can only be done accurately by the use of molecular tools such as amplification and sequencing of specific genomic regions. The RNA polymerase II largest subunit (RPB1), RNA polymerase II second largest subunit (RPB2), translation elongation factor-1a (TEF) and Bloc (Rehner *et al*., 2011), nuclear ribosomal internal transcribed spacer (ITS) and elongation factor 1- alpha (EF1-α) regions have all been used previously to study the *Beauveria* phylogeny (Rehner & Buckley, 2005). The analysis of sequences from specific genomic region and the study of phylogeny allow the identification of new fungal species and the reclassification of species groups such as *M. anisopliae* species which were reclassified under the four different species *M. anisopliae*, *M. pingshaense*, *M. robertsii* and *M. brunneum* (Bischoff *et al*., 2009). The phylogeny of the genus *Beauveria* was reanalysed using TEF, RPB1, RPB2 and Bloc by Rehner *et al.* (2011) which allowed the description of six new species of *Beauveria*, namely, *B. varroae*, *B. kipukae*, *B. pseudobassiana*, *B. asiatica*, *B. australis* and *B. sungii*.

1.8 Research aims and objectives

1.8.1 Aim

The aim of this research was to improve the understanding of the ecology of *B. bassiana* F418 in soil, with the ultimate goal to better the use of this biocontrol agent against the clover root weevil in New Zealand pastures.

1.8.2 Objectives

The overall objective of this research was to investigate the effect of some abiotic and biotic factors on *B. bassiana* F418 persistence and virulence in soil, aided by the use of *B. bassiana* F418 *gfp* transformants. In order to study the ecology of *B. bassiana* F418 in the complex soil environment, it was necessary to use a simplified soil microcosm of pasteurised or non-sterile soil to assess each abiotic or biotic factor independently. This was achieved through the following specific objectives:

- To determine if *B. bassiana* F418 *gfp* transformants were suitable as substitutes of *B. bassiana* F418 for ecological studies in soil.
- To determine the effects of some abiotic and biotic factors (soil moisture, temperature, peat content, presence of host, non-host or no insect, fertilisers and red clover plant) on *B. bassiana* F418 or *gfp* transformants persistence and virulence in soil.
- To determine the effects of selected environmental factors on *B. bassiana* F418 *gfp* transformant population dynamics in soil.

1.8.3 Thesis format

This thesis comprises seven chapters; five of which are experimental chapters. Each chapter includes an introduction, material and methods, results and a discussion section. The complete list of references is presented at the end of the thesis to keep repetition to a minimum.

Chapter 2
Characterisation of the green fluorescent protein transformants of *Beauveria bassiana* F418

2.1 Introduction

Understanding the ecology and dynamics of *Beauveria bassiana* F418 propagules in soil could help improve the use of this biocontrol agent against *S. lepidus* larvae in New Zealand pastures. Unfortunately, research on environmental factors affecting the fate of entomopathogenic fungi in soil is scarce (Jaronski, 2007).

The common technique of soil dilution plating onto semi selective media to estimate fungal populations in soil as CFU does not differentiate propagule types, e.g. between mycelia and conidia (Knudsen & Bin, 1990). Also, the use of semi selective media to recover entomopathogenic fungi is not specific enough to differentiate between strains of the same species of fungus, and therefore would not allow precise monitoring of only the introduced *B. bassiana* F418 as indigenous *B. bassiana* strains may also be present in the soil (Green & Jensen, 1995) as *B. bassiana* has a global distribution (Glare *et al*., 1993; Rehner & Buckley, 2005; Jaronski, 2007). The observation of different fungal propagule types in soil is essential to understand the fate and the population dynamics of the organism post-application, but this is difficult with a wild-type strain.

One solution to overcome these problems is to use a genetically engineered strain expressing the green fluorescent protein (*gfp*) (Bae & Knudsen, 2000). Such *gfp* transformants have been produced for *B. bassiana* F418 (Wu *et al.*, 2008). These *B. bassiana* F418 *gfp* transformants express the green fluorescence protein at all growth stages (mycelia and conidia) and also contain the hygromycin B resistance gene, allowing them to grow on media amended with hygromycin B whereas the wild-type F418 growth is inhibited on such selective media. These *B. bassiana* F418 *gfp* transformants would be a useful tool to monitor the fate and population dynamics of *B. bassiana* F418 in soil. However, before using *gfp* transformants as a substitute for *B. bassiana* F418, the comparison of their phenotype and genotype to the wild-type *B. bassiana* F418 is essential to ensure no

fundamental differences exist between the two which could impact their ability to infect potential hosts, survive, multiply and persist in the environment.

Beauveria bassiana F418 is a potential biocontrol of the host insect *S. lepidus*, however, the virulence of *B. bassiana* F418 and its *gfp* transformants was not tested against *S. lepidus* but against another host insect, *T. molitor*. This change of host was mainly due to the short period for field harvest of *S. lepidus* larvae which woud have constrained the timing of virulence bioassays and was also problematic due to the high number of host insects needed for the virulence bioassay (Philipps *et al.*, 2010). The availability of *T. molitor* larvae all year round and its susceptibility to *B. bassiana* made it an ideal host for virulence bioassay. Globally, *T. molitor* is a common host insect in virulence bioassays with entomopathogenic fungi (Dromph, 2003; Safavi *et al.*, 2007; Shah *et al*., 2005).

The aim of this chapter was to determine if *B. bassiana* F418 *gfp* tr1 and F418 *gfp* tr3 could be used as substitutes of *B. bassiana* F418 for ecological studies. The aim of this chapter was achieved by the following objectives: (i) to test whether *B. bassiana* F418 and its two transformants have the same germination rate, colony growth, conidial production, conidial size, virulence (LT_{50} and LD_{50}) against *T. molitor* larvae and the same conidiation level supported by *T. molitor* cadavers, (ii) to test whether there is antagonism or synergism between *B. bassiana* F418 and its two transformants during the infection of *T. molitor*, (iii) to test whether *B. bassiana* F418 *gfp* tr1 and F418 *gfp* tr3 emits fluorescence at the same level, (iv) to test the level of hygromycin B needed in the media to selectively inhibit *B. bassiana* F418 growth without altering F418 *gfp* transformants growth, (v) to test whether the two *B. bassiana* F418 *gfp* transformants are genetically identical to *B. bassiana* F418 on genetic regions used for strain identification and (vi) identify the location of the *gfp* cassette in the genome of the two *B. bassiana* F418 *gfp* transformants.

2.2 Methods

2.2.1 Fungi

2.2.1.1 Fungal strains

Three *Beauveria bassiana* strains used for this study were F418 and two *gfp* transformants (F418 *gfp* tr1 and F418 *gfp* tr3). *Beauveria bassiana* F418, *B. bassiana* F418 *gfp* tr1 and *B. bassiana* F418 *gfp* tr3 are referred as F418, F418 *gfp* tr1 and F418 *gfp* tr3 respectively in this chapter. The F418 strain was originally isolated from an adult clover root weevil, *S. lepidus*, collected in Europe and was obtained from AgResearch Insect Pathogens Culture Collection (Lincoln, New Zealand). The F418 *gfp* transformants were developed by Jiang Wu (Wu *et al*., 2008) using *Agrobacterium tumefaciens* Smith & Townsend (Rhizobiales: Rhizobiaceae) strain EHA105 and the plasmid pCAMBIA0300GFP containing the green fluorescent protein gene (*gfp*) isolated from the vector pCT74 (Lorang *et al*., 2001) and the hygromycin B resistance gene (*hph*) isolated from the vector pYT6. When the cultures were recovered from storage and prior to any experiments, a new single spore culture was developed for each transformant and the wildtype (method derived from El-Damir, 2006) to ensure homogeneity of the working stock as the previous procedures for storage and maintenance of the cultures was unknown. Mycelia and conidia from F418 *gfp* tr1 and F418 *gfp* tr3 can be visualised by fluorescence imaging (*gfp* gene) and can grow on specific media amended with hygromycin B due to their resistance to this antibiotic (*hph* gene). F418 *gfp* tr1 has one insertion of the *gfp-hph* cassette while F418 *gfp* tr3 has two insertions of the cassette (Wu *et al*., 2008).

2.2.1.2 Growth and production of conidia

The three strains were grown on 10 % Sabouraud dextrose agar (SDA) plates (Appendix A) for two weeks at 25 °C in the dark. These cultures were initiated from inoculum derived from a stock of single spore cultures (method derived from El-Damir, 2006) stored in a 1.2 mL screw cap tubes (LabCon, CA, USA) of 10 % glycerol at -80 °C. Conidia were harvested by scraping the media with an ethanol flamed loop and placed in a sterile 1.5 mL tube (Axygen,

CA, USA) containing 1 mL of 0.01 % Triton® X-100 (BDH, Poole, UK). Suspensions were mixed by vortexing and the concentration was determined using an improved Neubauer haemocytometer (double chamber, 0.1 mm depth). All suspensions were diluted to the desired concentration in sterile 0.01 % Triton® X-100.

Prior to each experiment, viability of conidia was confirmed by establishing germination rate. Conidial germination rate was assessed by plating 100 µL of a 10^8 conidia/mL suspension onto 10 % SDA. Plates were incubated for 24 h at 25 °C in the dark. After 24 h, two pieces of media (approximately 20 × 20 mm) were removed per plate and placed onto a microscope slide. A drop of lactophenol cotton blue stain (Merck, USA) was placed onto each piece of media and covered by a coverslip. Slides were examined under a light microscope (magnification 400 ×, Olympus BH2) and germination rate determined by examining at least 150 conidia on each piece of media; conidia with a germ tube equal or longer than the conidium diameter were scored as germinated (Hywel-Jones & Gillespie, 1990; Mander, 2006).

2.2.2 Comparison of phenotypic characteristics of wild-type and *gfp* transformants

2.2.2.1 Comparison of conidial germination rate

The objective of this experiment was to compare the germination rate of F418, F418 *gfp* tr1 and F418 *gfp* tr3 at different incubation time and temperature, to ensure that they are similar. Conidial germination rate was assessed by plating 100 µL of a 10^8 conidia/mL suspension onto commercially prepared SDA plates (Fort Richard Laboratories, Auckland, NZ) (Appendix A). Plates were incubated at 15, 20 and 25 ºC in the dark, with three replicate plates per temperature and strain using a randomized complete block design (RCBD). Germination rate was assessed after incubation for 14 and 24 h (plates at 20 and 25 ºC) and 24 and 48 h (for plates at 15 ºC), using the same protocol as described in section 2.2.1.2. The experiment comparing the germination rate of F418, F418 *gfp* tr1 and F418 *gfp* tr3 was repeated three times (October 2007, May 2009 and July 2009).

2.2.2.2 Comparison of colony growth rate

The objective of this experiment was to compare the colony growth rate of F418, F418 *gfp* tr1 and F418 *gfp* tr3 at different temperature, to ensure that they are similar. Colony growth rate was assessed by inoculating a sterile 6 mm Ø filter paper disc (grade 3, qualitative, Whatman, UK) with 10 µL of a 10^8 conidia/mL suspension and placing it in the centre of a commercially prepared SDA plate. Plates were incubated at 15, 20 and 25 ºC in the dark, with six replicates per temperature and strain using a RCBD. Colony diameter was measured at two axes at right angles to each other at 3 d intervals for 15 d (Shah *et al.*, 2005) using a standard ruler. The initial marking was made at 3 d and the growth was counted from this initial growth point. The experiment comparing F418, F418 *gfp* tr1 and F418 *gfp* tr3 was repeated twice (May and July 2009).

2.2.2.3 Comparison of conidial production

The objective of this experiment was to compare the conidial production of F418, F418 *gfp* tr1 and F418 *gfp* tr3 at different temperature, to ensure that they are similar. Conidial production was assessed by plating 100 µL of a 10^8 conidia/mL suspension onto commercially prepared SDA plates. Plates were incubated at 15, 20 and 25 ºC in the dark, with three replicates per temperature and strain using a RCBD. After incubation for 15 d, two 10 mm Ø discs were cut from each replicate plate using a sterile core borer; each disc was placed in a 10 mL centrifuge tube (LabServ, NZ) with 5 mL of sterile 0.01 % Triton® X-100. The tube was vortexed for approximately 1 min to dislodge conidia from the media. The resulting suspension was diluted if necessary and the conidial concentration determined using an improved Neubauer haemocytometer. The entire experiment was repeated twice (May and July 2009). In July 2009, plates were incubated at 15 and 20 ºC only due to growth and conidiation issues observed at 25 ºC.

2.2.2.4 Comparison of conidial size

The objective of this experiment was to compare the conidial size of F418, F418 *gfp* tr1 and F418 *gfp* tr3 at different temperature, to ensure that they are similar.

Conidial size was assessed using conidia harvested from plates used to determine conidial yields (section 2.2.2.3). A 10 mm Ø agar disc was cut from each replicate plate and placed in a 10 mL centrifuge tube with 5 mL of sterile 0.01 % Triton® X-100. The tube was vortexed

for approximately 1 min and 20 µL of the suspension was placed onto a microscope slide; 10 µL of lactophenol cotton blue stain was mixed with the suspension before covering with a coverslip. Slides were examined using light microscopy (magnification 400 ×, Olympus BX50). The length and width of at least 50 conidia were measured using the arbitrary distance measuring tool of the Olysia microscope imaging software (Olympus). The experiment was repeated twice using F418, F418 *gfp* tr1 and F418 *gfp* tr3 strains (May and July 2009).

2.2.2.5 Comparison of virulence against *Tenebrio molitor* larvae

The objective of this experiment was to compare the virulence (LT_{50}) of different concentrations of F418, F418 *gfp* tr1 and F418 *gfp* tr3 against *T. molitor* larvae, to ensure that they are similar.

Virulence bioassays were carried out against 4-5th instar *T. molitor* (Biosuppliers, Auckland, New Zealand). In routine laboratory tests, *T. molitor* is commonly used as a representative host insect. This is due to its availability and susceptibility to the pathogen. Filter paper (grade 1, 10 cm Ø, Whatman, UK) were fitted into a 9 cm Ø Petri dish (LabServ, NZ) and 1 mL of a 10^4 or 10^6 conidia/mL suspension pipetted evenly over the filter paper. One mL of 0.01 % Triton® X-100 only was added to the filter paper for the control. Five *T. molitor* larvae were then added to each Petri dish. The dishes were held at room temperature (20 °C ± 2), with 18 replicate dishes (separated into 6 different blocks) per fungus and fungal dose using a RCBD. There were three replicates of control dishes per block. Each block of plates was arranged on moistened paper towel and covered by a plastic sheet to maintain conditions of high relative humidity (>95 %) throughout the experiment. Mortality was recorded daily for a maximum of 15 d. Dead larvae were left in the Petri dish to promote fungal outgrowth and conidiation to confirm that death resulted from infection by the applied *B. bassiana* strains. The experiment was repeated once using all three strains (May 2009).

2.2.2.6 Comparison of virulence and conidia production on *T. molitor* following inoculation with single or mixed strains of *B. bassiana*

The objective of this experiment was to ensure that F418, F418 *gfp* tr1 and F418 *gfp* tr3 have similar virulence (LT_{50}) against *T. molitor* and similar conidia production when used individually or as a mix. Cruz *et al*. (2006) observed antagonism/synergism when using

mixtures of genetically different strain of *B. bassiana* compared to the virulence of each individual strain whereas no difference in virulence was observed when the strains in the mixtures were similar genetically. Studying the virulence and conidia production of single and mixed strains of *B. bassiana* could bring confirmation that the strains are similar to each other.

Virulence bioassays were carried out against 4-5th instar *T. molitor*, using fungal suspensions containing 10^7 or 10^5 conidia/mL, prepared from one fungal strain, or a combination of two strains in the following ratios: 50:50, 25:75, 75:25.

Batches of five or six *T. molitor* larvae were immersed in a 5 mL fungal suspension for 10 s and excess liquid was removed by vacuum filtration. Treated larvae were transferred to a dry filter paper for 2 min. Control larvae were immersed in sterile 0.01 % Triton® X-100 only. After exposure to conidia, larvae were held individually in 6-well plates on a sterile filter paper disc moistened with 150 µL of sterile distilled water (SDW). Each 6 well-plate contained larvae from an identical treatment. Plates were incubated at room temperature (20 °C ± 2) with three replicates plates per treatment separated into three different blocks. Each block was held in a closed plastic bag containing a moistened paper towel to maintain conditions of high relative humidity (>95 %).

Four experiments were completed, one with F418 and F418 *gfp* tr1 mixed suspensions at 10^7 conidia/mL (in April 2009) and at 10^5 conidia/mL (in March 2010) and one with F418 and F418 *gfp* tr3 mixed suspensions at 10^5 conidia/mL (in June 2009) and at 10^7 conidia/mL (in March 2010). Batches of six larvae were used in the experiments set up in 2009 while batches of five larvae were used in the experiments set up in 2010.

Mortality was recorded daily for 15 d. Dead larvae were removed from the plates, surface sterilised by dipping in 70 % ethanol for 1 min followed by three washes of 1 min each in SDW. Once surface sterilised, cadavers were held in 10 mL tubes containing a piece of moistened sterile cotton to promote conidiation. Cadavers were incubated for two-three weeks at room temperature (20 °C ± 2).

After incubation, 5 mL of sterile 0.01 % Triton® X-100 was added to each tube containing the cadavers. The tube was vortexed for approximately 2 min to dislodge conidia from the cadaver. The resulting suspension was diluted if necessary and the conidial concentration determined using an improved Neubauer haemocytometer. Suspensions were also plated (100 µL) onto specific selective media (10 % SDA+A+Hyg and 10 % SDA+A, Appendix A) to determine the proportion of transformant/wild-type strain conidia in the suspension. Plates were incubated at 20 ºC in the dark for two weeks and CFU were counted. Colonies growing

on 10 % SDA+A+Hyg medium were from F418 *gfp* transformants only while colonies growing on 10 % SDA+A were from both wild-type F418 and F418 *gfp* transformants. Therefore CFU numbers for wild-type F418 were calculated by subtracting the number of CFU growing on 10 % SDA+A+Hyg from the number of CFU growing on 10 % SDA+A.

2.2.2.7 Comparison of virulence and production of conidia on different sizes of *T. molitor* larvae

The objective of this experiment was to compare the virulence (LT_{50} and LD_{50}) and the conidial production of different concentrations of F418, F418 *gfp* tr1 and F418 *gfp* tr3 against two sizes of *T. molitor* larvae, to ensure that they are similar. The virulence bioassay was carried out using two sizes of *T. molitor* larvae, small size S (approximately 30 mg) and large size L (approximately 110 mg) using 10^2, 10^3, 10^4 and 10^5 conidia/mL suspension of either F418, F418 *gfp* tr1 or F418 *gfp* tr3. Batches of four larvae of the same size were immersed for 10 s in a 5 mL conidial suspension and excess liquid removed by vacuum filtration followed by placing the larvae for 2 min on a sterile filter paper. The protocols for incubation of the larvae, surface sterilisation of the cadaver and conidia counts were the same as those used in the previous experiment (section 2.2.2.6) apart from incubation of cadavers which was done in 60 mL container instead of a 10 mL tube.

2.2.2.8 Comparison of fluorescence from F418 *gfp* tr1 and F418 *gfp* tr3

The objective of this experiment was to determine if the fluorescence emitted by F418 *gfp* tr1 and F418 *gfp* tr3 was similar. Suspensions of F418 *gfp* tr1 and F418 *gfp* tr3 were prepared at 10^8 conidia/mL and 20 µL of each suspension was placed onto microscope slides. Slides were examined using fluorescence microscopy (magnification 400 ×, Olympus BX50, excitation filter Olympus U-MWIB3, software Olympus Olysia and DP2-BSW, camera Olympus DP12 and DP72, Olympus Corp., Tokyo, Japan) to determine the intensity of the fluorescence emitted by each transformant.

2.2.2.9 Specific media for growth of F418 *gfp* transformants

The objective of this experiment was to determine the concentration of hygromycin B needed in 10 % SDA medium to selectively inhibit F418 growth without altering F418 *gfp* transformants' growth. Ten percent SDA medium was autoclaved (Appendix A), then amended with 0, 100, 200 or 400 mg/L of hygromycin B (50 mg/mL, Invitrogen, USA). F418 and the *gfp* transformants were plated onto the different media using 100 µL of suspension containing 10^8 conidia/mL per plate. Plates were incubated at 25 °C in the dark for two weeks and were then observed for fungal growth. The appropriate level of hygromycin B needed in the media was one which inhibited completely F418 growth as well as did not change the growth of the *gfp* transformants (colony morphology and number) compared to the media without hygromycin B.

2.2.3 Molecular identification of the F418 *gfp* transformants strains and localisation of the *hph-gfp* cassette insertion

The objectives of these experiments were to confirm that the two *gfp* transformants were truly transformants of *B. bassiana* and to potentially explain the differences between the *gfp* transformed strains by localising the position of the *gfp* cassette in the genomes.

2.2.3.1 DNA extraction

Mycelia of *B*. *bassiana* F418, F418 *gfp* tr1 and tr3 were grown by plating conidia from each strain on a sterile piece of cellophane overlain onto a 10 % SDA plate. Plates were incubated at 25ºC in the dark for 3 d before DNA extraction. Mycelia were harvested from the cellophane using a sterile spatula and transferred into 1.2 mL sterile tubes. The tubes were dipped into liquid nitrogen and mycelia were ground using a sterile plastic pestle. DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Netherlands) and Gentra Puregene DNA isolation kit (formerly Gentra Systems, Mineapolis, USA, now Qiagen). DNA quality and concentration was assessed by gel electrophoresis and spectrophotometry (NanoDrop, Thermo scientific, USA).

2.2.3.2 PCR amplification and sequencing for molecular identification

DNA amplification of the nuclear ribosomal internal transcribed spacer region ITS and elongation factor 1-alpha (EF1-α) regions were performed using the corresponding primers listed in Table 2.1. Each PCR contained 1 × PCR reaction buffer with 1.5 mM $MgCL_2$ (Roche, Basel, Switzerland), 200 µM of each dNTP (Roche), 10 pM of each primer (Invitrogen, Life Technologies, CA, USA), 1 µL bovine serum albumin (10 mg/mL, New England BioLabs Incorporation, MA, USA), and 1.25 U Taq Expand High Fidelity PCR System (Roche) and 10 ng of isolated DNA in a total volume of 50 µL. A DNA-free control was included in every PCR to ensure absence of contamination. PCRs were performed in an icycler (Bio-Rad, CA, USA) using the thermal settings corresponding to each set of primers described in Table 2.2. Amplified products were visualised on 1 % agarose gels (Appendix A) alongside a 100 bp molecular ladder (TrackIt, Invitrogen) run at 10 V/cm for 45 min followed by staining for 10 min in ethidium bromide (0.5 µg per mL of 1 × TAE), destained in water and photographed under UV light using VersaDoc™ Imaging System (Model 3000, Bio-Rad, CA, USA).

Selected PCR products were sequenced in both directions (Bio-Protection Research Centre Sequencing Facility, Lincoln University, New Zealand) and aligned using ClustalW (Thompson *et al*., 1994) in Bioedit software (Ibis Biosciences, CA, USA) to obtain consensus sequences. Phylogenetic trees were constructed using F418, F418 *gfp* tr1 and F418 *gfp* tr3 consensus sequences and sequences from other *Beauveria* and *Isaria* species from Rehner *et al.* (2011). All sequences were aligned using MUSCLE (Edgar, 2004) in Mega 5 (Tamura *et al.*, 2011). Phylogenetic trees for ITS and EF1-α gene region sequences were constructed using the Neighbour-Joining method (Saitou & Nei, 1987) using the Mega 5 package (Tamura *et al.*, 2011). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein, 1985).

Table 2.1 List of primers used for the molecular identification of the strains

Gene	Primer	5' Sequence 3'	Direction	Source
ITS	ITS4	TCCTCCGCTTATTGATATGC	F	White *et al*., 1990
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	R	
EF 1-α	1577F	CARGAYGTBTACAAGATYGGTGG	F	Rehner & Buckley,
EF 1-α	2218R	ATGACACCRACRGCRACRGTYTG	R	2005

Table 2.2 Thermal settings used for the molecular identification of the strains

Gene	Primers	Thermal settings
ITS	ITS4-ITS5	94 °C, 2 min 30 cycles (94 °C, 30 s, 56 °C, 30 s, 72 °C, 45 s) 72 °C, 10 min
EF 1-α	1577F-2218R	94 °C, 2 min 10 cycles (94 °C, 30 s, 60-50 °C, 30 s (decreasing by 1 °C each cycle, 72 °C, 1 min) 30 cycles (94 °C, 30 s, 50 °C, 30 s, 72 °C, 1 min) 72 °C, 10 min

2.2.3.3 Localisation of the *gfp* cassette in the genome of the two transformants using TAIL-PCR

The TAIL-PCR technique (Liu & Huang, 1998) was used to identify the region flanking the *gfp-hph* cassette inserted in the genome of F418 *gfp* tr1 and F418 *gfp* tr3. The first TAIL-PCR was done using DNA from F418 *gfp* tr1 and F418 *gfp* tr3 (extraction method in section 2.2.3.1) with the set of TAIL-PCR primers *gfp* FWD1, *gfp* FWD 2 and *gfp* FWD3 (Invitrogen, New Zealand) and the arbitrary degenerate (AD) primers AD1, AD2 and AD3 (Invitrogen, New Zealand) (Table 2.3). The *gfp* FWD primers set was designed from the *gfp-hph* cassette sequenced (Carpenter M., personal communication) from the vector p0300gfp used to create the F418 *gfp* transformant.

2.2.3.3.1 Primary TAIL-PCR

Primary TAIL-PCR were performed in a total volume of 25 µL containing 1 × PCR reaction buffer with 20 mM $MgCL_2$ (Roche, Basel, Switzerland), 100 µM of each dNTP (Roche), 10 pM of each specific primer (*gfp* FWD1) (Invitrogen, Life Technologies, CA, USA), 10 pM of arbitrary degenerate (AD) primer (Invitrogen) and 1.25 U FastStart Taq DNA polymerase (Roche) and 5 ng of isolated DNA in a total volume of 25 µL. A negative, DNA-free control was included in every PCR run to ensure absence of contamination. An icycler (Bio-Rad, CA, USA) thermocycler was used with the thermal conditions for primary PCR described in Table 2.4. Amplified products were visualised as described in section 2.2.3.2.

2.2.3.3.2 Secondary TAIL-PCR

Secondary TAIL-PCR were performed using the same protocol as for the primary TAIL-PCR except that the DNA template was a 1/40 dilution of the PCR product obtained following the primary PCR and the specific primer was *gfp* FWD 2. Thermal conditions for secondary PCR are described in Table 2.4. Amplified products were visualised as described in section 2.2.3.2.

2.2.3.3.3 Tertiary TAIL-PCR

Tertiary TAIL-PCR was performed using the same protocol as for the primary TAIL-PCR except that the DNA template was a 1/10 dilution of the PCR product obtained following the secondary PCR and the specific primer was *gfp* FWD 3. Thermal conditions for tertiary PCR are described in Table 2.4. Amplified products were visualised as described in section 2.2.3.2. Unique bands were sequenced at the Bio-Protection Research Centre Sequencing Facility (Lincoln University, Lincoln, New Zealand).

2.2.3.3.4 Additional primers, sequencing and analysis

A new set of TAIL-PCR primers (NOS term, Table 2.3) was designed close to the end of the sequence obtained during the TAIL-PCR with *gfp* FWD primers. TAIL-PCR with NOS term primers set (Invitrogen, New Zealand) was carried out on F418 *gfp* tr1 and F418 *gfp* tr3 following the same protocol than above (with *gfp* FWD primer set). Unique bands obtained were sequenced at the Bio-Protection Research Centre Sequencing Facility (Lincoln University, Lincoln, New Zealand).

From the sequence obtained with NOS term primers, a new set of TAIL-PCR primers were designed (CaMV 35S primers, Table 2.3). TAIL-PCR with CaMV 35S primers set was carried out on F418 *gfp* tr1 and F418 *gfp* tr3 following the same protocol than above (with *gfp* FWD primer set).

Selected PCR products were sequenced (Bio-Protection Research Centre Sequencing Facility, Lincoln University, New Zealand) and aligned using Bioedit software (Ibis Biosciences, CA, USA). Sequences inside and outside the *gfp-hph* cassette were compared using the programme BLAST from NCBI (Altschul *et al*., 1990) to find a match to known genes.

Table 2.3 List of primers used for TAIL-PCR

Gene	Primer	5' Sequence 3'	Source
	AD1	NTCGASTWTSGWGTT	Liu & Huang
	AD2	NGTCGASWGANAWGAA	(1998)
	AD3	WGTGNAGWANCANAGA	
gfp	*gfp* FWD 1	GTCCAGGAGCGCACCATC	This study
gfp	*gfp* FWD 2	GGGCACAAGCTGGAGTACAA	
gfp	*gfp* FWD 3	GAACGGCATCAAGGTGAAC	
NOS	NOS term 1	GAATCCTGTTGCCGGTCTT	
NOS	NOS term 2	TGCATGACGTTATTTATGAGATGG	
NOS	NOS term 3	TGATTAGAGTCCCGCAATTA	
NOS	NOS term FWD	CAATTCCACACAACATACGA	
CaMV 35S	CaMV 35S 2	CGAGTTTCTCCATAATAATGTGT	
CaMV 35S	CaMV 35S 3	AATCCAGATCCCCCGAATTA	
CaMV 35S	CaMV 35S 4	AATGTGTTATTAAGTTGTCTAAGCGTC	

Table 2.4 Thermal settings used for TAIL-PCR

Reaction	Step	Number of cycles	Thermal conditions
Primary	1	1	92 °C (3 min), 95 °C (1 min)
	2	5	94 °C (30 s), 58 °C (1 min), 72 °C (2 min)
	3	1	94 °C (30 s), 25 °C (2 min), ramping to 72 °C over 2 min, 72 °C (2 min)
	4	15	94 °C (30 s), 58 °C (1 min), 72 °C (2 min)
			94 °C (30 s), 58 °C (1 min), 72 °C (2 min)
			94 °C (30 s), 44 °C (1 min), 72 °C (2 min)
	5	1	72 °C (5 min)
Secondary	1	1	94 °C (2 min)
	2	12*	94 °C (30 s), 58 °C (1 min), 72 °C (2 min)
			94 °C (30 s), 58 °C (1 min), 72 °C (2 min)
			94 °C (30 s), 45 °C (1 min), 72 °C (2 min)
	3	1	72 °C (5 min)
Tertiary	1	1	94 °C (2 min)
	2	20**	94 °C (40 s), 45 °C (1 min), 72 °C (2 min)
	3	1	72 °C (5 min)

(*18 cycles instead of 12 for some PCR, **40 cycles instead 20 for some PCR)

2.2.4 Statistical analysis

Germination rate and conidial production data were log_{10} transformed to achieve a normal distribution essential when using analysis of variance (ANOVA) (Olsen, 2003). Virulence was expressed as the LT_{50} (days) and the LC_{50} (log_{10} of concentrations) determined by probit analysis after correction of the mortality data based on the control group mortality using Abbott's formula (Abbott, 1925) when necessary. LT_{50} values obtained after probit analysis were log_{10} transformed. Analyses of variance (ANOVA) were carried out to compare the germination rates (log_{10}), colony growth rates, conidial production (log_{10}), conidial size and the virulence (LT_{50} values, LC_{50} values and slope values in log_{10}) with treatment factors being the treatments (i.e. fungus, temperature of incubation, time of incubation, fungal concentration and insect size) with a linear polynomial contrast specified where appropriate. Linear regression analyses were carried out to test the correlation between the initial ratio of F418 *gfp* transformants/F418 conidia used to infect the insects and the final ratio of conidia produced by F418 *gfp* transformants/F418 on the cadavers. Significance was evaluated at $P < 0.05$ for all analyses and mean separation was executed by the unprotected LSD test. All data analyses were performed using the statistical software Genstat version 12.2 (VSN International). All statistical results tables are presented in Appendix C.

2.3 Results

2.3.1 Comparison of the phenotypical characteristics

2.3.1.1 Conidial germination rate

There was no significant difference in germination rates among the three isolates after incubation at 20 and 25 ºC for 24 h or after incubation at 15 ºC for 48 h (Table 2.5); germination rates ranged from 96.6 to 100 % throughout. Conidia from F418 *gfp* tr3 germinated at a significantly faster rate compared to both F418 and F418 *gfp* tr1 at the shorter incubation times (24 h at 15 ºC and 14 h at 20 ºC). Conidia from F418 *gfp* tr1 germinated at a significantly slower rate compared to F418 wild-type after 14 h incubation at

25 ºC; differences were not significant at the other incubation times and temperatures. Germination rates for all strains increased with longer incubation times and higher temperatures.

Table 2.5 Germination rate (%) (Log_{10} values) of F418, F418 *gfp* tr1 and F418 *gfp* tr3 on SDA at different incubation times and temperatures. An asterix indicates a significant difference from other values within the same column (P < 0.05).

	Incubation time & temperature					
Fungus	15 °C & 24 h (**Log_{10}**)	15 °C & 48 h (**Log_{10}**)	20 °C & 14 h (**Log_{10}**)	20 °C & 24 h (**Log_{10}**)	25 °C & 14 h (**Log_{10}**)	25 °C & 24 h (**Log_{10}**)
F418	34.3 (**1.536**)	99.4 (**1.997**)	4.3 (**0.635**)	97.5 (**1.989**)	53.5 (**1.728**)	97.2 (**1.988**)
F418 *gfp* tr1	32.1 (**1.507**)	98.4 (**1.993**)	4.6 (**0.664**)	96.6 (**1.985**)	42.6 (**1.630***)	100 (**2.000**)
F418 *gfp* tr3	52.1 (**1.717***)	98.5 (**1.993**)	14.2 (**1.153***)	97.3 (**1.988**)	63.1 (**1.800**)	98.1 (**1.992**)
LSD (P < 0.05)	**0.089**	**0.089**	**0.089**	**0.089**	**0.089**	**0.089**

2.3.1.2 Colony growth rate

The colony growth rate of F418 *gfp* tr3 at 15 and 20 ºC (0.99 and 1.37 mm/d respectively) was significantly slower than F418 (1.11 and 1.74 mm/d respectively), 11 and 21 % slower, respectively (Table 2.6 and Fig. 2.1). There was no difference in colony growth rate between F418 *gfp* tr3 and F418 at 25 ºC. F418 *gfp* tr1 colony growth was not different from F418 at any temperature. Colony growth rate for all strains increased at higher temperatures.

Table 2.6 Colony growth rate (mm/d) of F418, F418 *gfp* tr1 and F418 *gfp* tr3 on SDA over 15 d incubation at 15, 20 and 25 °C. Growth measures with an asterix are significantly different from other values within the same column (P < 0.05).

Fungus	Incubation temperature		
	15 °C	20 °C	25 °C
F418	**1.1**	**1.7**	**2.6**
F418 *gfp* tr1	**1.1**	**1.8**	**2.6**
F418 *gfp* tr3	**1.0***	**1.4***	**2.6**
LSD (P < 0.05)	**0.12**	**0.12**	**0.12**

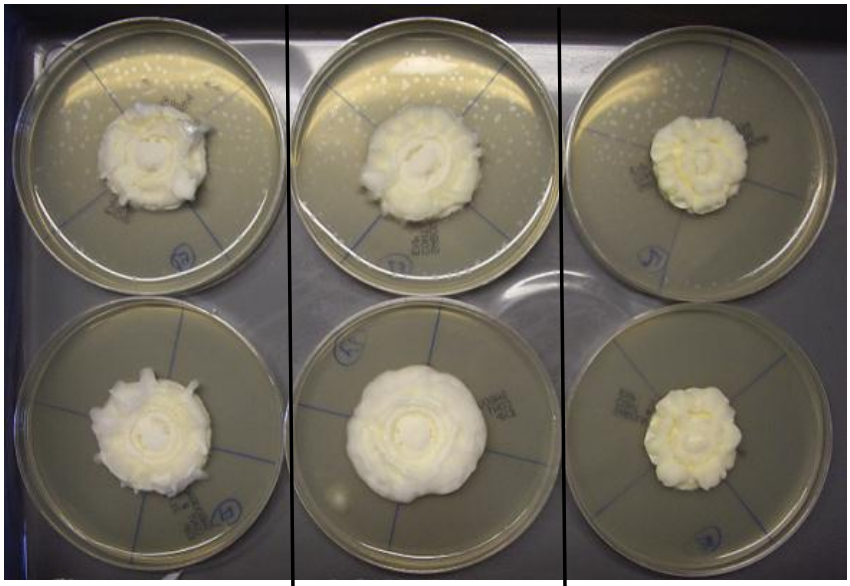

Figure 2.1 F418, F418 *gfp* tr1 and F418 *gfp* tr3 colonies on SDA plates at 20 °C after 15 d.

2.3.1.3 Conidial production

The production of conidia from a 10 mm Ø disc of F418 *gfp* tr1 on SDA was not significantly different from production obtained from F418 at all temperatures tested (Table 2.7). Productions of conidia from F418 *gfp* tr3 were not different from those obtained from F418 at 15 and 20 ºC, but were significantly lower at 25 ºC.

Temperature of incubation had a significant effect on conidial production. The average productions for all fungi were significantly higher at 20 ºC with 3.36×10^8 conidia/disc (=8.53 in $\log_{10}$) than at 15 ºC with 1.03×10^8 conidia/disc (=8.01 in $\log_{10}$) (LSD [P < 0.05] =0.14).

On some plates incubated at 25 ºC, the growth morphology of *B. bassiana* was different to that observed at lower temperatures. Characteristically, *B. bassiana* F418 and *gfp* transformants grew as a thin mycelial mat which conidiated profusely. On the abnormal plates, the fungus grew as a dense mycelial mat and conidiation was sparse. While the ratio of abnormal/normal plates for each strain was not recorded, the phenomenon seemed to occur at random among the three strains and was not restricted to a single strain.

Table 2.7 Conidial production of F418, F418 *gfp* tr1 and F418 *gfp* tr3 from a 10 mm Ø SDA disc after incubation at 15, 20 and 25 °C for 15 d. Numbers followed by an asterix are significantly different from other values within the same column ($P < 0.05$).

	Incubation temperature					
	15 °C		20 °C		25 °C	
Fungus	Conidia yield	Log_{10} value	Conidia yield	Log_{10} value	Conidia yield	Log_{10} value
F418	8.71×10^7	**7.94**	3.72×10^8	**8.57**	7.41×10^7	**7.87**
F418 *gfp* tr1	1.02×10^8	**8.01**	3.63×10^8	**8.56**	7.08×10^7	**7.85**
F418 *gfp* tr3	1.20×10^8	**8.08**	2.82×10^8	**8.45**	3.72×10^7	**7.57***
LSD ($P < 0.05$)		**0.24**		**0.24**		**0.30**

2.3.1.4 Conidial size

Conidia produced by F418 *gfp* tr1 and F418 *gfp* tr3 were not different in size from the wild-type at any of the incubation temperatures (Table 2.8). The average size of conidia ranged from 2.51 to 2.79 µm in length. There was no significant difference in conidia size between incubation temperatures.

Table 2.8 Conidia size (length) of F418, F418 *gfp* tr1 and F418 *gfp* tr3 on SDA media after incubation at 15, 20 and 25 °C for 15 d. Numbers followed by an asterix are significantly difference from other values within the same column (P < 0.05).

Fungus	Incubation temperature		
	15 °C	20 °C	25 °C
F418	2.79	2.64	2.67
F418 *gfp* tr1	2.76	2.71	2.55
F418 *gfp* tr3	2.69	2.76	2.51
LSD (P < 0.05)	0.16	0.16	0.21

2.3.1.5 Virulence against *T. molitor* larvae

The virulence of F418 *gfp* tr1 and F418 *gfp* tr3 against 4-5th instar of *T. molitor* was not significantly different from F418 at either concentration (10^4 and 10^6 conidia/mL) (Table 2.9 and Fig. 2.2). LT_{50} values ranged from 10.49 to 10.86 d for all fungi applied at 10^4 conidia/mL, and from 7.18 to 7.29 d for all fungi applied at 10^6 conidia/mL. The LT_{50} values were significantly lower for all fungi when applied at the higher concentration, i.e. applied at 10^6 conidia/mL gave a mean LT_{50} value of 7.22 d (= 0.859 in $\log_{10}$) compared to 10.67 d (= 1.028 in $\log_{10}$) (LSD (P < 0.05) = 0.013) when applied at 10^4 conidia/mL. The overall goodness of fit for the probit model was good for both doses. None of the insects in the control treatment died, so Abbott's formula was not used to correct the data.

Figure 2.2 Photographs of *T. molitor* larvae from the comparison of virulence of F418, F418 *gfp* tr1 and F418 *gfp* tr3 experiment, with larvae from the control on the left and larvae from the treatment supporting *B. bassiana* conidiation on the right.

Table 2.9 Virulence (LT_{50}) and regression slope of two conidial concentrations of F418, F418 *gfp* tr1 and F418 *gfp* tr3 against 4^{th}-5^{th} instar *T. molitor* at 20 ± 2 °C. Values followed by an asterix are significantly different from other values within the same column (P < 0.05).

Fungus	10^4 conidia/mL			10^6 conidia/mL		
	LT_{50} (d)	Log_{10} LT_{50}	Regression slope	LT_{50} (d)	Log_{10} LT_{50}	Regression slope
F418	10.49	**1.021**	**1.01**	7.19	**0.857**	**1.67**
F418 *gfp* tr1	10.66	**1.028**	**1.09**	7.18	**0.856**	**2.05**
F418 *gfp* tr3	10.86	**1.036**	**0.95**	7.29	**0.863**	**2**
LSD (P < 0.05)		**0.023**	**0.844**		**0.023**	**0.844**
d.f.		180			144	
Chi^2		35.85			30.44	

2.3.1.6 Virulence and conidial production following inoculation of *T. molitor* with single or mixed strain suspensions

2.3.1.6.1 F418 *gfp* tr1 and F418 single or mixed suspension

There was no difference in LT_{50} values and the regression slopes between any of the single or mixed strain combinations of F418 *gfp* tr1 and F418 applied at 10^5 and 10^7 conidia/mL (Table 2.10). The LT_{50} values ranged from 6.89 to 9.10 d for all fungal combinations applied at 10^5 conidia/mL and from 5.98 to 6.26 d for all fungal combinations applied at 10^7 conidia/mL. The overall goodness of fit for the probit model was good for both doses. There was 20 % control mortality in the first block in the experiment using 10^5 conidia/mL, and the data for the treatment were corrected using Abbott's formula; there was no control mortality in the other two blocks in the same experiment. No mortality occurred in the controls in any of the three blocks in the experiment using 10^7 conidia/mL so Abbott's formula was not used.

Table 2.10 Virulence (LT_{50}) and regression slope of two conidial concentrations of F418 and its transformant F418 *gfp* tr1 applied individually or as a mixed suspension against 4^{th}-5^{th} instar *T. molitor* at 20 ± 2 °C. Values followed by an asterix are significantly different from other values within the same column (P < 0.05).

Fungus	10^5 conidia/mL			10^7 conidia/mL		
	LT_{50} (d)	Log_{10} LT_{50}	slope	LT_{50} (d)	Log_{10} LT_{50}	slope
F418	7.35	**0.866**	**3.55**	6.26	**0.796**	**1.22**
F418/F418 *gfp* tr1 25:75	6.89	**0.838**	**3.81**	6.20	**0.793**	**1.21**
F418/F418 *gfp* tr1 50:50	7.46	**0.873**	**2.32**	6.17	**0.790**	**2.27**
F418/F418 *gfp* tr1 75:25	6.89	**0.838**	**3.71**	5.98	**0.777**	**2.33**
F418 *gfp* tr1	9.10	**0.959**	**1.02**	6.14	**0.788**	**1.18**
LSD (P < 0.05)		**0.170**	**3.87**		**0.038**	**1.83**
d.f.		120			105	
Chi^2		22.23			32.24	

There was no difference in the yield of conidia produced on *T. molitor* larvae infected by either single or mixed strain combinations (Table 2.11). The average conidia production obtained per insect was 2.1×10^7 and 1×10^7 conidia following inoculation with 10^5 and 10^7 conidia/mL, respectively.

The ratio of F418/F418 *gfp* tr1 conidia produced from *T. molitor* larvae infected by mixed strains suspension compared to the initial ratio used to infect the insects showed that there was no correlation between the initial and the final ratio obtained.

Table 2.11 Production of conidia on *T. molitor* larvae killed by single or mixed strain suspensions of F418 and F418 *gfp* tr1. Values followed by an asterix are significantly different from other values within the same column (P < 0.05).

Fungus	10^5 conidia/mL		10^7 conidia/mL	
	Conidia/insect	Log_{10} conidia	Conidia/insect	Log_{10} conidia
F418	2.06×10^7	**7.31**	9.73×10^6	**6.99**
F418/F418 *gfp* tr1 25:75	1.91×10^7	**7.28**	1.07×10^7	**7.01**
F418/F418 *gfp* tr1 50:50	1.95×10^7	**7.29**	1.01×10^7	**7.01**
F418/F418 *gfp* tr1 75:25	1.99×10^7	**7.30**	8.79×10^6	**6.94**
F418 *gfp* tr1	2.63×10^7	**7.42**	1.14×10^7	**7.06**
LSD (P < 0.05)		**0.52**		**0.46**

2.3.1.6.2 F418 *gfp* tr3 and F418 single or mixed suspension

There was no difference in LT_{50} values and the regression slopes between any of the single or mixed strains combinations of F418 *gfp* tr3 and F418 applied at 10^5 and 10^7 conidia/mL (Table 2.12). The LT_{50} values ranged from 9.89 to 11.25 d for all fungal combinations applied at 10^5 conidia/mL and 4.86 to 5.48 d for all fungal combinations applied at 10^7 conidia/mL. The overall goodness of fit for the probit model was good for both doses. There was 16.7 % mortality in the controls contained in the first block in the experiment using 10^5 conidia/mL, and 20 % in the third block; data were therefore corrected using Abbott's formula. No mortality occurred in the control in the second block. There was 20 % control mortality in the second block in the experiment using 10^7 conidia/mL, and the data for the treatment were corrected using Abbott's formula. No mortality occurred in the control in the other two blocks in the same experiment.

Table 2.12 Virulence (LT_{50}) and regression slope of two conidial concentrations of F418 and its transformant F418 *gfp* tr3 applied as single or mixed strain suspensions against 4^{th}-5^{th} instar *T. molitor* at 20 ± 2 °C. Values followed by an asterix are significantly different from other values within the same column (P < 0.05).

Fungus	10^5 conidia/mL			10^7 conidia/mL		
	LT_{50} (d)	Log_{10} LT_{50}	Slope	LT_{50} (d)	Log_{10} LT_{50}	Slope
F418	10.45	**1.019**	**0.31**	5.48	**0.739**	**1.11**
F418/F418 *gfp* tr3 25:75	9.89	**0.995**	**0.93**	5.16	**0.713**	**2.58**
F418/F418 *gfp* tr3 50:50	11.25	**1.051**	**0.35**	4.86	**0.687**	**5.05**
F418/F418 *gfp* tr3 75:25	10.47	**1.020**	**0.38**	5.07	**0.705**	**4.88**
F418 *gfp* tr3	9.14	**0.961**	**2.07**	5.26	**0.721**	**2.50**
LSD (P < 0.05)		**0.144**	**1.81**		**0.068**	**5.44**
d.f.		195			90	
Chi^2		69.96			15.73	

There was no difference in the yield of conidia produced on *T. molitor* larvae infected by either single or mixed strain combinations of F418 and F418 *gfp* tr3 applied at 10^5 conidia/mL (Table 2.13).

The yield of conidia obtained from *T. molitor* larvae infected by F418/F418 *gfp* tr3 at the 75:25 ratio was significantly lower than yields obtained from all other treatments. The average number of conidia obtained per insect was 3.58×10^7 and 1.15×10^8 conidia following treatment with 10^5 and 10^7 conidia/mL suspensions, respectively.

The ratio of F418/F418 *gfp* tr3 conidia produced from *T. molitor* larvae infected by mixed strain suspensions compared to the initial ratio used to infect the insects showed that there was no correlation between the initial and the final ratio obtained.

Table 2.13 Production of conidia on *T. molitor* larvae killed by single or mixed strain suspensions of F418 and F418 *gfp* tr3. Values followed by an asterix are significantly different from other values within the same column (P < 0.05).

Fungus	10^5 conidia/mL		10^7 conidia/mL	
	Conidia/insect	Log_{10} conidia	Conidia/insect	Log_{10} conidia
F418	2.62×10^7	**7.42**	3.16×10^8	**8.50**
F418/F418 *gfp* tr3 25:75	4.06×10^7	**7.61**	9.93×10^7	**8.00**
F418/F418 *gfp* tr3 50:50	2.84×10^7	**7.45**	1.21×10^8	**8.08**
F418/F418 *gfp* tr3 75:25	3.40×10^7	**7.53**	2.67×10^7	**7.43***
F418 *gfp* tr3	5.69×10^7	**7.76**	1.96×10^8	**8.29**
LSD (P < 0.05)		**0.34**		**0.55**

2.3.1.7 Virulence (LT_{50} and LC_{50}) and conidial production following inoculation of two different sizes of *T. molitor* with different concentration of conidia

Calculated LT_{50} values for F418 *gfp* tr3 after treatment of small and large *T. molitor* larvae with 10^4 and 10^5 conidia/mL were not significantly different from those obtained for F418 for each dose and insect size (Table 2.14 top section).

The calculated LT_{50} for F418 *gfp* tr1 was significantly higher (12.80 d = 1.107 in log_{10}) than the LT_{50} for F418 (10.33 d = 1.014 in log_{10}) when large larvae were treated with 10^4 conidia/mL; there was no difference in values for the small larvae. Comparative values for 10^5 conidia/mL were not significantly different for small and large larvae.

The LT_{50} value obtained for all fungi at 10^4 conidia/mL against both sizes of *T. molitor* (12.16 d = 1.085 in log_{10}) was significantly higher than for 10^5 conidia/mL (8.44 d = 0.926 in log_{10}) (LSD [P < 0.05] = 0.033).

The overall goodness of fit for the probit model was good. There was no difference among the regression slopes for all three fungi for each dose and larval size (Table 2.14 lower section) although the regression slopes for all fungi were significantly different between the 10^4 and 10^5 conidia/mL doses (0.946 and 1.108 respectively with P < 0.05 = 0.148) and between the small and large larvae (1.150 and 0.904 respectively with P < 0.05 = 0.148).

Table 2.14 Virulence (LT_{50}) (top section) and regression slopes (lower section) for two conidial concentrations of F418, F418 *gfp* tr1 and F418 *gfp* tr3 against two sizes of *T. molitor* larvae at 20 ± 2 °C. Values followed by an asterix are significantly different from other values within the same column (P < 0.05).

Fungus	10^4 conidia/mL small larvae		10^4 conidia/mL large larvae		10^5 conidia/mL small larvae		10^5 conidia/mL large larvae	
	LT_{50} (d)	Log_{10} LT_{50}	LT_{50} (d)	Log_{10} LT_{50}	LT_{50} (d)	Log_{10} LT_{50}	LT_{50} (d)	Log_{10} LT_{50}
F418	12.57	**1.099**	10.33	**1.014**	7.79	**0.891**	9.85	**0.994**
F418 *gfp* tr1	13.69	**1.136**	12.80	**1.107***	8.16	**0.912**	8.65	**0.937**
F418 *gfp* tr3	11.96	**1.078**	11.87	**1.075**	7.84	**0.894**	8.53	**0.931**
LSD(P < 0.05)		**0.082**		**0.082**		**0.082**		**0.082**
	d.f.		429		Chi^2		101.2	

Fungus	10^4 conidia/mL small larvae	10^4 conidia/mL large larvae	10^5 conidia/mL small larvae	10^5 conidia/mL large larvae
	Log_{10} slope	Log_{10} slope	Log_{10} slope	Log_{10} slope
F418	**1.052**	**0.802**	**1.196**	**0.835**
F418 *gfp* tr1	**1.105**	**1.004**	**1.447**	**0.993**
F418 *gfp* tr3	**0.888**	**0.825**	**1.214**	**0.966**
LSD (P < 0.05)		**0.362**		

F418 *gfp* tr1 and F418 *gfp* tr3 LC_{50} values and regression slopes calculated for both small and large *T. molitor* were not significantly different from F418 (Table 2.15). The mean LC_{50} value for all three strains was 6.28×10^3 conidia (=3.798 in log_{10}) for small larvae and 1.18×10^4 conidia (=4.071 in log_{10}) for large larvae (LSD [P < 0.05] = 0.424); these values are not significantly different.

Table 2.15 Virulence (LC_{50}) of F418, F418 *gfp* tr1 and F418 *gfp* tr3 against two sizes of *T. molitor* larvae at 20 ± 2 °C. LC_{50} values followed by an asterix are significantly different from other values within the same column (P < 0.05).

Fungus	Large larvae			Small larvae		
	LC_{50}	Log_{10} LC_{50}	Log_{10} slope	LC_{50}	Log_{10} LC_{50}	Log_{10} slope
F418	1.38×10^4	**4.141**	**0.17**	4.95×10^3	**3.695**	**0.52**
F418 *gfp* tr1	1.25×10^4	**4.097**	**0.51**	9.98×10^3	**3.999**	**0.60**
F418 *gfp* tr3	9.44×10^3	**3.975**	**0.48**	5.01×10^3	**3.700**	**0.35**
LSD (P < 0.05)		**0.734**	**0.774**		**0.734**	**0.774**

There were no differences in conidia yields obtained following treatment of both small and large larvae with 10^4 conidia/mL of F418 *gfp* tr1, F418 *gfp* tr3 and F418 (Table 2.16). The yield obtained following treatment of small larvae with 10^5 conidia/mL of F418 *gfp* tr3 (5.60×10^7 conidia/cadaver) was significantly higher than F418 (1.09×10^7 conidia/cadaver) and F418 *gfp* tr1 (9.33×10^6 conidia/cadaver). The yield of F418 *gfp* tr3 conidia following treatment of large larvae with 10^5 conidia/mL (8.81×10^7 conidia/cadaver) was significantly higher than F418 (1.95×10^7conidia).

The mean yield for all three fungi applied at 10^4 conidia/mL to both larval sizes (2.97×10^7 conidia =7.473 in log_{10}) was not significantly different from yields obtained following treatment with 10^5 conidia/mL (2.86×10^7 conidia = 7.456 in log_{10}) (P < 0.05) = 0.102). The mean yield of conidia from all fungi with both test concentrations was significantly lower from small larvae (1.75×10^7 conidia =7.243 in log_{10}) than large larvae (4.86×10^7 conidia = 7.687 in log_{10}) (P < 0.05) = 0.102).

Table 2.16 Production of conidia on small and large *T. molitor* larvae killed after treatment with 10^4 or 10^5 conidia/mL of F418, F418 *gfp* tr1 and F418 *gfp* tr3. Numbers followed by an asterix are significantly different from other values within the same column (P < 0.05).

Fungus	10^4 conidia/mL small larvae		10^4 conidia/mL large larvae		10^5 conidia/mL small larvae		10^5 conidia/mL large larvae	
	Spore yield	Log_{10} spore	Spore yield	Log_{10} Spore	Spore yield	Log_{10} spore	Spore yield	Log_{10} spore
F418	1.50×10^7	**7.175**	6.40×10^7	**7.806**	1.09×10^7	**7.037**	1.95×10^7	**7.289**
F418 *gfp* tr1	2.26×10^7	**7.355**	3.53×10^7	**7.548**	9.33×10^6	**6.970**	5.61×10^7	**7.749**
F418 *gfp* tr3	1.49×10^7	**7.174**	6.07×10^7	**7.783**	5.60×10^7	**7.748***	8.81×10^7	**7.945***
LSD (P < 0.05)		**0.499**		**0.499**		**0.499**		**0.499**

2.3.1.8 Fluorescence emitted by F418 *gfp* tr1 and F418 *gfp* tr3

When F418 *gfp* tr1 and F418 *gfp* tr3 conidia suspensions were observed under fluorescence microscopy at the same concentration, the level of fluorescence produced by F418 *gfp* tr3 conidia was approximately twice as large as the fluorescence from F418 *gfp* tr1 conidia.

2.3.1.9 Choice of specific media for the growth of F418 *gfp* transformants

Wild-type F418 grew on 10 % SDA media and on 10 % SDA amended with 100 mg/L of hygromcyin B, although the growth was partially inhibited on the hygromycin medium. The growth of F418 was totally inhibited on media amended with 200 and 400 mg/L of hygromcyin B. F418 *gfp* tr1 and F418 *gfp* tr3, however, grew on all of the hygromycin B-amended media as well as the 10 % SDA-only medium.

2.3.2 Genomic characteristics of the F418 *gfp* transformants

2.3.2.1 Phylogenetic comparison of F418 and its transformants F418 *gfp* tr1 and F418 *gfp* tr3 with other *Beauveria* sp.

Both EF1-α and ITS phylogenic trees (Figs 2.3 and 2.4 respectively) showed that *B. bassiana* F418 and its transformants F418 *gfp* tr1 and F418 *gfp* tr3 all clustered in a single clade belonging to strains of *B. bassiana*. The boot strap value at the branch point between the clade containing *B. bassiana* F418 and F418 *gfp* transformants and the other isolates of *B. bassiana* was 65 for the EF1-α tree and 95 for the ITS tree. There was a strong support for the separation between F418, F418 *gfp* tr1, F418 *gfp* tr3 and the other strains of *B. bassiana* in the ITS tree whereas the support was weaker in the EF1-α tree. The pairwise distance analysis showed that there were more nucleotide differences between F418, F418 *gfp* tr1, F418 *gfp* tr3 and the other strains of *B. bassiana* in the ITS tree than in the EF1-α tree.

The pairwise distance analysis also showed that there were no nucleotide differences between F418, F418 *gfp* tr1 and F418 *gfp* tr3 sequences for EF1-α and ITS regions.

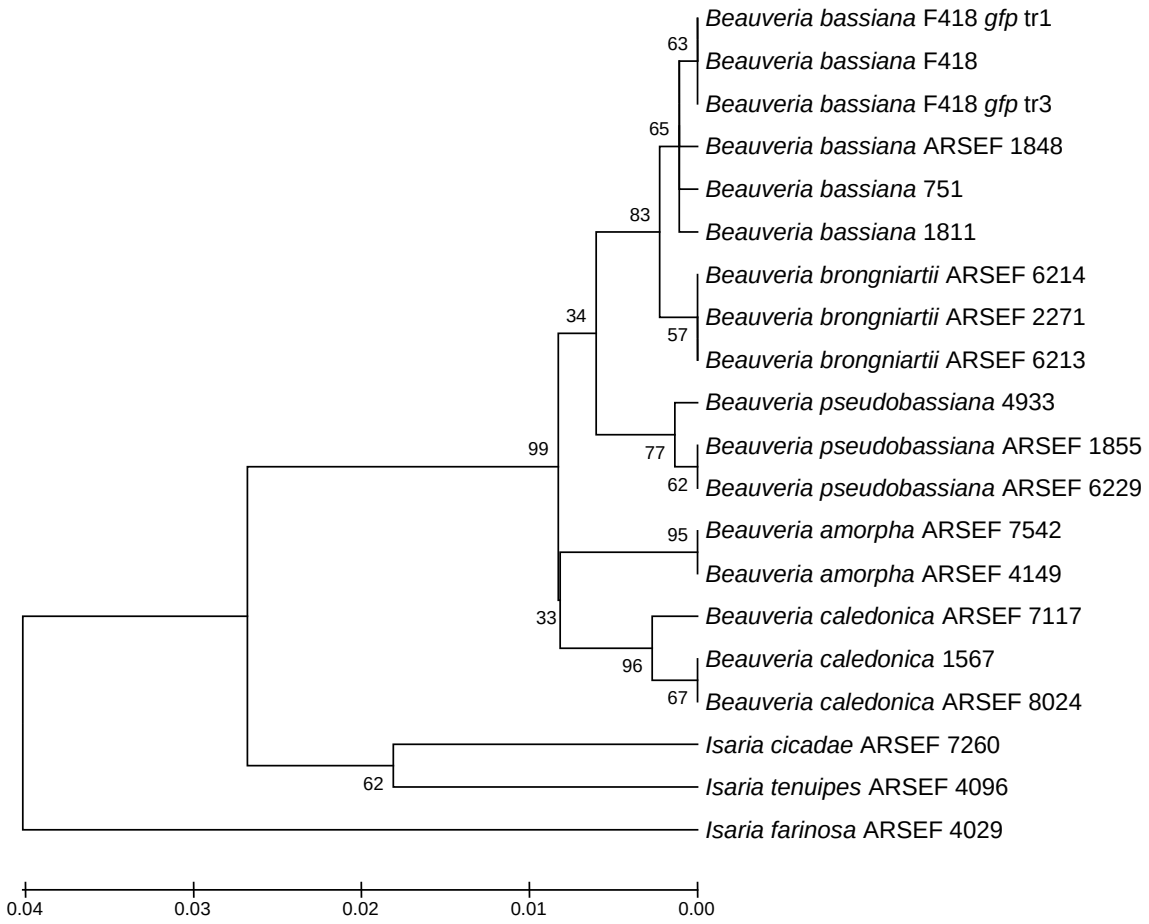

Figure 2.3 Phylogeny of *Beauveria* illustrating species relationships inferred from neighbour joining analyses of EF1. *Isaria* species were used as an outgroup. The numbers on the branches indicate boostrap value (%) for 1000 replicates.

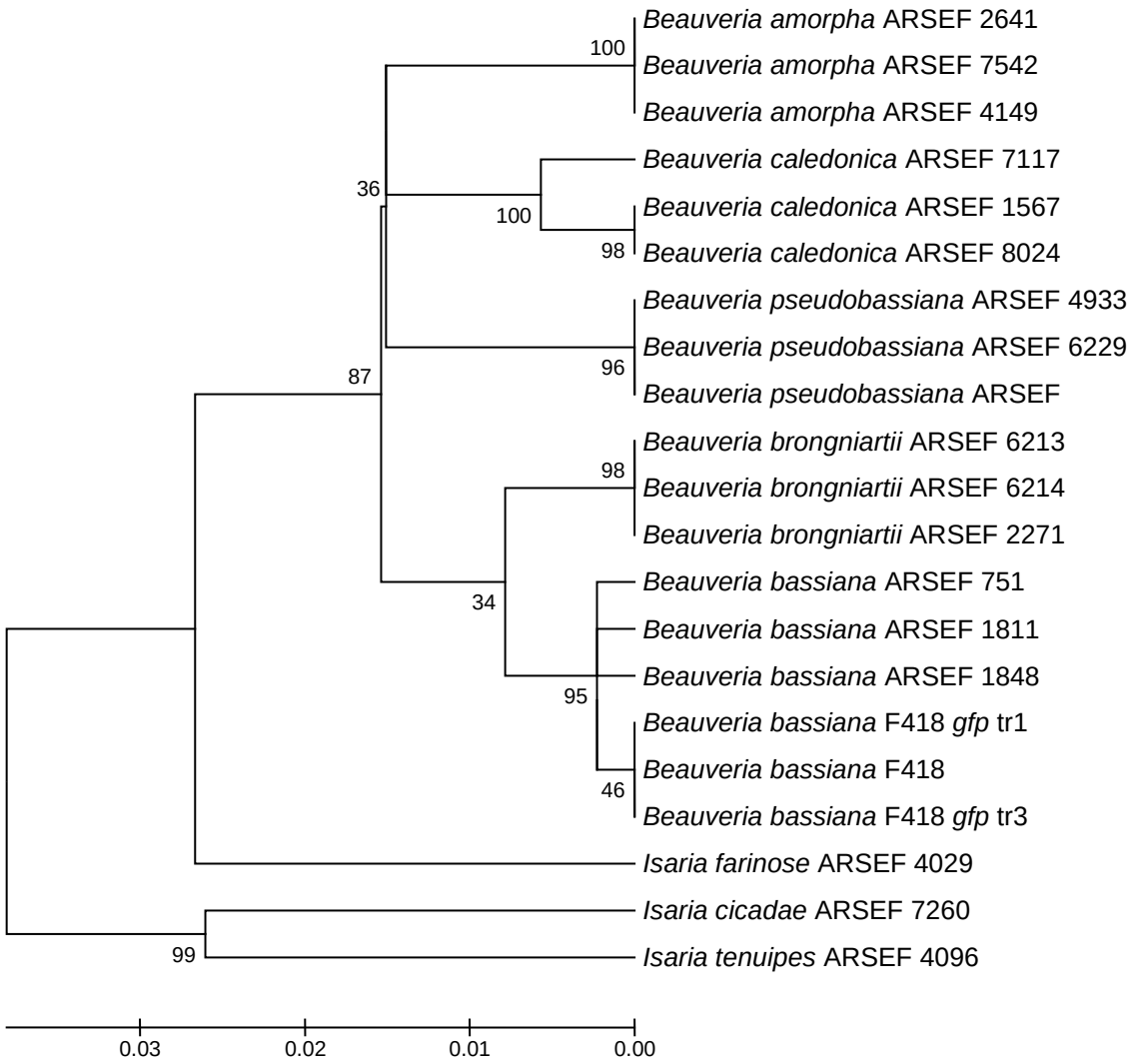

Figure 2.4 Phylogeny of *Beauveria* illustrating species relationships inferred from neighbour joining analyses of ITS. *Isaria* species were used as an outgroup. The numbers on the branches indicate boostrap value (%) for 1000 replicates.

2.3.2.2 Localisation of the *gfp* cassette in the genome of F418 transformants

The TAIL-PCR on F418 *gfp* tr1 and F418 *gfp* tr3 using the *gfp* FWD, NOS term and CAMV35S produced the expected multiple bands in primary and secondary reactions. The desired single bands were produced in several of the tertiary reactions. An example of TAIL-PCR using CAMV35S primers is shown in Fig. 2.5, where combination with AD1 and AD3 produced single tertiary products of 250 and 150 bp, respectively. The results of other TAIL-PCR are not shown.

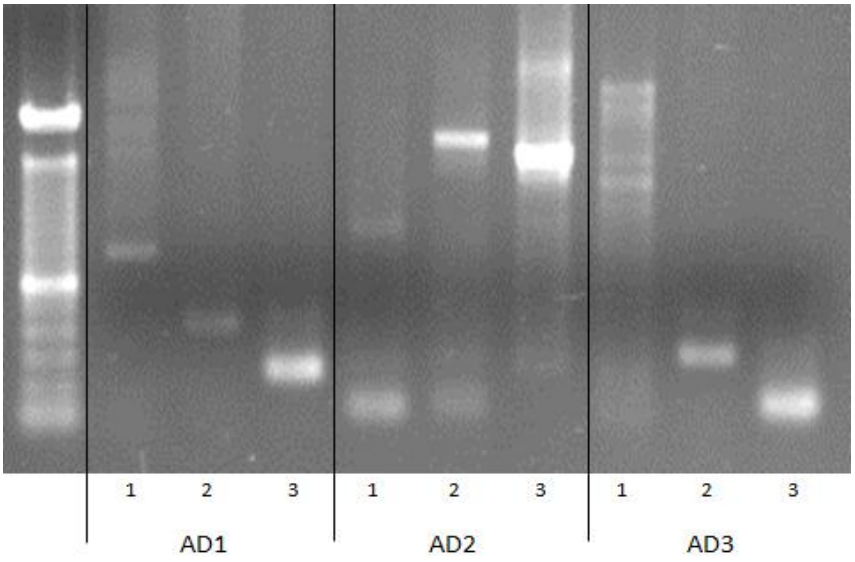

Figure 2.5 Example of 1 % agarose gel of TAIL-PCR of F418 *gfp* tr1 with CaMV35S primers set and AD1, AD2, AD3 primers after primary (1), secondary (2) and tertiary (3) TAIL-PCR. First lane is the 100 bp DNA ladder.

The consensus sequence of the aligned tertiary PCR products of F418 *gfp* tr1 and F418 *gfp* tr3 were used to reconstruct the insert DNA of 1023 bp (Appendix B) which matched sGFP gene, NOS terminator and CaMV35S present in the original *hph-gfp* cassette. For each transformant the sequence beyond the common part of *hph-gfp* cassette was unique (Appendix B). The sequences were 1050 and 714 bp, for F418 *gfp* tr1 and F418 *gfp* tr3, respectively. Only one unique sequence beyond the *hph-gfp* cassette was found for F418 *gfp* tr3 even though it had two insertions in the genome. The translated 1050 bp product from F418 *gfp* tr1 had weak homology to several proteins of which the best match (13 % coverage, E value = 0.70) was to a coiled-coil domain of *Pongo abelii* Lesson (Primates: Hominidae) (Sumatran orang-utan). The translated 714 bp product produced from F418 *gfp* tr3 had some homology to hypothetical proteins from several organisms of which the first named product was zinc finger protein 281 (16 % coverage, E value = 5×10^{-5}) from the common rabbit *Oryctolagus cuniculus* L. (Lagomorpha: Leporidae).

2.4 Discussion

2.4.1 Germination rate comparison

Conidia from both F418 *gfp* tr1 and F418 *gfp* tr3 germinated at rates that were similar to the wild-type F418 at all temperatures tested when incubated for long times. However, it was evident that conidia from F418 *gfp* tr3 germinated at a significantly faster rate at all temperatures tested when germination was assessed at shorter incubation times and conidia from F418 *gfp* tr1 germinated more slowly at 25 °C compared to F418 after 14 h incubation.

El-Damir (2006) found that single spore isolates of *B. bassiana* 5672 had significant differences in their germination rates compared to the multispore isolate they were obtained from. *Beauveria bassiana* 5672 single spore isolate 1 (5672-1) had a 98 % germination rate at 30 °C and 100 % RH whereas *B. bassiana* 5672 multispore isolate germination rate was 72 %. El-Damir (2006) showed that not only there were differences in germination rates between different strains of *B. bassiana*, there also were some differences within the single spore isolates originated from the same isolate. Similarly to El-Damir (2006), F418, F418 *gfp* tr1 and F418 *gfp* tr3 used in this study were originated from single spore isolates for each of the strains. Therefore, the differences in germination rate observed with F418 *gfp* tr3 and F418 *gfp* tr1 might not have been observed using a different single spore isolate for each strain and the variations observed are similar to natural variations observed by El-Damir (2006). Also, the *hph-gfp* cassette insertions in F418 *gfp* tr3 and F418 *gfp* tr1 might have disrupted some genes involved in the biochemical pathway of germination, leading to the differences observed and this hypothesis cannot be ruled out.

The germination rates of all three strains increased with increasing time and temperature between 15 and 25 °C which is consistent with other reports (e.g. Hywel-Jones & Gillespie, 1990; Luz & Fargues, 1997; Ekesi *et al.* 1999; Mander, 2006).

2.4.2 Colony growth comparison

There was no difference in the growth rate of F418 *gfp* tr1 compared to F418. This result is in accordance with Wu *et al.* (2008) who also found similar colony growth rates between F418 *gfp* tr1 and F418 at 26 °C. The growth rate of F418 *gfp* tr3, however, was significantly slower than F418 at 15 and 20 °C. Wu *et al.* (2008) found that F418 *gfp* tr2 had a significantly slower colony growth compared to F418, showing variations between the different F418 *gfp* transformants. Bae & Knudsen (2000) also found a slower colony growth rate for their *T. harzianum gfp* cotransformant ThzID1-M3 compared to their wild-type ThzID1. The slow colony growth of F418 *gfp* tr3 is not explained by differences in germination rate.

The *hph-gfp* cassette insertions into the F418 *gfp* tr3 genome might have disrupted genes involved in the development of mycelia, therefore delaying the colony growth compared to the wild-type. As F418 *gfp* tr3 contains two insertions of the *hph-gfp* cassette, it is possible that the increase in genome size and the extra metabolic load due to the production of *gfp* delayed mycelia production in F418 *gfp* tr3. However, the genome size of *B. bassiana* is approximately 41 Mb (Viaud *et al*., 1996) and the *hph-gfp* cassette is 4.2 kb, so the insertion of two cassettes is an increase of 0.02 % of the total genome weight, a very small difference.

Hohmann (2010) found that one of the *Trichoderma hamatum* LU592 *gfp* transformants used in his study grew slower than the wild-type on soil extract agar but had the same growth rate when cultured on PDA. Colony growth of F418 and its *gfp* transformants was compared on SDA only, therefore it is possible that there would be no difference if cultured on different media.

All colony growth rates increased with increasing temperature which is similar to results presented by Hallsworth & Magan (1999) and Ekesi *et al.* (1999).

2.4.3 Conidia production and conidial size comparison

Production of conidia by F418 *gfp* tr1 and F418 *gfp* tr3 was similar to F418, and no differences were detected in conidial size across the strains. Production of conidia increased for all 3 test strains between 15 and 20 °C, and yields were lower at 25 than at 15 °C. These results concur with those of Mander (2006) who used the same F418 strain.

The only significant difference observed in the current study was for F418 *gfp* tr3 which produced fewer conidia at 25 °C compared to F418. This difference might be due to periodically-observed abnormal growth at 25 °C on SDA. Although the incidence of abnormal plates appeared to occur at random, a higher incidence for F418 *gfp* tr3 would have resulted in lower production levels overall. But this is a difficult hypothesis to confirm since the ratio of abnormal/normal plates was not recorded and comparison of conidial yields at 25 °C was not repeated in the second comparison of phenotypic traits in July 2010 due to the observed abnormalities. Wu *et al.* (2008) found that production of conidia by F418 *gfp* tr1 was significantly lower than F418 at 26 °C and reported that F418 *gfp* tr1 showed 'fluffy' growth. However, there is no mention of the rate of occurrence of such abnormal colonies in F418 *gfp* tr1 or whether any other *gfp* transformant or the wild-type ever exhibited similar growth abnormalities. Their conidia production comparison study was set up on PDA media; production of conidia differs on different experimental media. When F418, F418 *gfp* tr1 and F418 *gfp* tr3 were grown on 10 % SDA or 10 % PDA at 25 °C, no abnormal growth occurred.

Also, El-Damir (2006) observed some significant differences in conidia production between single spore isolates and multispore isolate of *B. bassiana* 5672. For example, conidia production of *B. bassiana* 5672-3 was not different from *B. bassiana* 5672 at 100 % RH and 15 °C, whereas there was a significant difference at 20 °C. El-Damir (2006) showed that there was some natural variations between single spore isolates originated from the same multispore isolate *B. bassiana* 5672. These natural variations could also explain the difference in conidia production of F418 *gfp* tr3 at 25 °C.

Since the transformants will be used at 15 °C in soil experiments, a poor nutrient environment compared to SDA media, the difference in conidial production observed for F418 *gfp* tr3 on SDA at 25 °C is not likely to be an issue.

2.4.4 Virulence against *T. molitor* larvae

F418 *gfp* tr1 and F418 *gfp* tr3 had similar virulence (as measured by LT_{50} and LC_{50} values) to F418. Although each experiment comparing the virulence of F418 and its two transformants was only repeated once, three different experiments quantifying and comparing different aspects of virulence were carried out. In all three trials, no differences were detected in virulence between the wild-type and its transformants with the exception of the calculated LT_{50} values for F418 *gfp* tr1 and F418 when bioassayed against large

T. molitor larvae at 10^4 conidia/mL. All three strains thus appear to have similar characteristics of virulence.

El-Damir (2006) observed significant differences in virulence against the western flower thrips, *Frankliniella occidentalis* Pergande (Thynasoptera: Thripidae), between *B. bassiana* 5672 multispore isolate and its different single spore isolates. His study showed that there were some natural variations between single spore isolates originated from the same multispore isolate *B. bassiana* 5672. These natural variations could explain the difference of virulence observed with F418 *gfp* tr1 in one experiment.

Comparative assays carried out by Cantone & Vanderberg (1999) and Cao *et al.* (2007) revealed no differences in virulence between *gfp* transformants and wild-type strains of *Paecilomyces fumosoroseus* (Wize) A.H.S. Br. & G. Sm. (Ascomycota: Eurotiales) and *M. anispoliae* respectively. However, Inglis *et al.* (2000) found that *gfp* transformants of *M. anisopliae* var. *acridum* (= *M. acridum*) CG423 exhibited 'delayed virulence' against *Anticarsia gemmatalis* Hubner (Lepidoptera: Noctuidae) compared to the wild-type parent strain, although differences were attributed to the number of subcultures the *gfp* transformants went through on artificial media (which is known to affect the virulence; Fargues & Robert, 1983), as opposed to it being a result of the transformation.

In all experiments, LT_{50} values decreased with increasing conidial concentrations which is in agreement with other studies (Feng & Johnson, 1990; Feng *et al.*, 1990; Jones *et al.*, 1996).

There were no apparent antagonistic or synergistic interactions when F418 and each of its transformants were used in a mixed inoculum against *T. molitor* larvae. Similarly, Cruz *et al.* (2006) showed that there was no difference in virulence against the coffee berry borer, *Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae), when inoculated with a mixture of *B. bassiana* strains when the strains were genetically similar.

2.4.5 Conidial production on *T. molitor* larvae

Production of conidia on *T. molitor* larvae infected with F418 *gfp* tr1 or F418 *gfp* tr3 was similar to production on larvae infected with F418. However, production of conidia on larvae infected with a mixture of F418/F418 *gfp* tr3 at a 75:25 ratio using 10^7 conidia/mL was significantly lower than for all other treatments using 10^5 conidia/mL. Production on small

and large *T. molitor* larvae after inoculation with F418 *gfp* tr3 using 10^5 conidia/mL was significantly higher than for F418 at the same concentration.

It was noted that the environment in which dead larvae were left to conidiate had an impact on the level of conidiation. In an experiment not presented in this thesis, dead larvae were left in 1.5 mL tubes to conidiate, but the level of conidiation was neither high nor homogenous, probably due to a lack of space around the dead larvae and the lack of constant high humidity. Therefore, 10 mL tubes containing a moistened cotton ball then 60 mL containers with moistened filter paper were used in subsequent experiments. However, there were also drawbacks to using both of these methods. The 10 mL tubes were narrow and there was little free space around the dead larvae which could have impacted conidiation. When using the 60 mL container, the piece of sterile filter paper dried faster in some containers than others, perhaps due to differences in the seals around the lid of the container. This could have affected the relative humidity within the container leading to the observed differences in conidiation. Therefore the differences in conidia production on *T. molitor* cadavers observed could have been due to the environment in which cadavers were left for fungal conidiation or to natural variations as observed by El-Damir (2006).

Conidial production for all fungi was higher on large *T. molitor* larvae compared to small larvae when the same conidial concentration was used, probably due to the larger surface area available on large larvae for growth of mycelia and conidiation to occur.

The ratio of F418/F418 *gfp* transformant obtained from *T. molitor* cadavers after co-inoculation with a mixed conidial suspension was not correlated to the ratio of the strains in the initial inoculum. This result indicated that there was no competition between the two strains. Chandler *et al.* (1993) infected *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) with a mix of two different strains of *L. lecanii* (formerly *Verticillium lecanii*) (1.72 and 19.79) and could not find any conidia from the strain 1.72 produced on the cadavers of the white flies showing a strong antagonism between the two strains. A conserved ratio of the mixed strains of F418/F418 *gfp* transformant could have been expected since there is neither antagonism nor synergism between the two strains showed by the LT_{50}. However, deviation from this ratio may be due to variables such as the site of penetration by each conidium, the local environment within the body of the insect (affecting blastospore production) (Altre & Vandenberg, 2001) and site where mycelia exits. It would require an in depth study of the infection process inside the cadaver to determine the outcomes and reasons, which is outside the scope of the present study.

2.4.6 Fluorescence comparison

Fluorescence of F418 *gfp* tr3 conidia was twice as bright as that of conidia from F418 *gfp* tr1. F418 *gfp* tr3 has two insertions of the *hph-gfp* cassette which might lead to the production of twice the amount of green fluorescent proteins and would explain the brighter fluorescence observed for F418 *gfp* tr3. However, Cantone & Vandenberg (1999) compared the fluorescence of different *gfp* transformants of *P. fumosoroseus* and found that the different levels of fluorescence were not correlated to the number of *gfp* copies integrated but could be due to differences in cassette position in the chromosomes (Spellig *et al.*, 1996; Vanden Wymelenberg *et al.*, 1997). The lower brightness of F418 *gfp* tr1 might be an issue in the study of propagule dynamics in soil and the brighter transformant would likely be a better choice, with greater fluorescence aiding detection.

The fluorescence could have been quantified using spectrophotometry (Cantone & Vanderberg, 1999) to give an accurate measure of the difference observed; however since the fluorescence difference between the transformants was easily 2 fold, a visual examination was sufficient to conclude that F418 *gfp* tr3 was more suitable for subsequent experiments.

2.4.7 Genomic studies

The identities of F418, F418 *gfp* tr1 and F418 *gfp* tr3 were confirmed as *Beauveria bassiana*. All strains belong to the same clade in both phylogenetic trees based on analyses using the EF1-α and ITS genes region. EF1-α and ITS regions were chosen because they have been used extensively by Rehner *et al.* (2011) and therefore there is a large number of reliable sequences available for different *Beauveria* species, which helped to build a comprehensive phylogenetic tree. F418, F418 *gfp* tr1 and F418 *gfp* tr3 were genetically identical on the EF1-α and ITS regions sequenced. This genomic study confirmed that F418 *gfp* tr1 and F418 *gfp* tr3 were transformed isolates of *B. bassiana* F418.

The localisation of the *hph-gfp* cassette in the genome of both F418 *gfp* transformants could help understanding differences in *gfp* expression, or differences in colony growth; it would also add data to the relatively limited information available about insertion sites, particularly in fungi. DNA of outside the *hph-gfp* cassette has been successfully sequenced for both *gfp* transformants and the closest match was a coiled-coil domain found in orang-utan for F418 *gfp* tr1 and zinc finger protein 281 in rabbit for F418 *gfp* tr3. These homologies were

weak with only 13 and 16 % of the sequences covered respectively. A complete genome sequence of *B. bassiana* and other relative fungi would be necessary to find the genes surrounding the *hph-gfp* cassette but this is not available yet.

We had anticipated finding two different DNA sequences outside the *hph-gfp* cassette for F418 *gfp* tr3 as there are two insertions inside its genome (Wu *et al.*, 2008). Unfortunately only one sequence was successfully isolated and analysed. More time could have been spent to find the location of the other *hph-gfp* cassette and longer sequences outside the cassettes for both transformants, but determining the location of the *hph-gfp* cassette in the genome of the F418 *gfp* transformants was not the main goal of this project and therefore the question was only partially answered.

2.4.8 Conclusions

The following conclusions can be drawn from the results presented in this chapter:

- F418 *gfp* tr1 and F418 *gfp* tr3 are both phenotypically similar to the wild-type F418 in general.
- The few differences obtained between F418 *gfp* tr1 and F418 *gfp* tr3 and the wild-type were within the range of natural variations observed between single spore isolates issued from a same multispore isolate.
- F418 *gfp* tr1 was, based on these phenotypical comparisons, more similar to F418 than F418 *gfp* tr3 was.
- F418 *gfp* tr1 and F418 *gfp* tr3 did not present antagonistic or synergetic behaviour with F418 during the infection of *T. molitor* larvae.
- F418 *gfp* tr3 expressed brighter fluorescence than F418 *gfp* tr1.
- A quantity of 200 mg/L of hygromycin B in 10 % SDA medium creates a selective media inhibiting the growth of F418 whithout altering the growth of F418 *gfp* tr1 and F418 *gfp* tr3.
- F418 *gfp* tr1 and F418 *gfp* tr3 and F418 identities were confirmed as being *B. bassiana* isolates using phylogenetic trees and F418 *gfp* tr1 and F418 *gfp* tr3 were confirmed as being transformed isolates of F418.

- The localisation of the *gfp-hph* cassette in the genome of F418 *gfp* tr1 and F418 *gfp* tr3 was not completed.
- Although F418 *gfp* tr1 presented more similarities to F418 than F418 *gfp* tr3, F418 *gfp* tr3 has the advantage of expressing brighter fluorescence. Therefore, both F418 *gfp* tr1 and F418 *gfp* tr3 can be used in ecological studies in soil as substitutes for the wild-type F418 to examine the fate, dynamics and persistence of the fungus.

Chapter 3
Effect of abiotic and biotic factors on persistence and virulence of *Beauveria bassiana* F418 and F418 *gfp* tr1 in soil

3.1 Introduction

Beauveria bassiana F418 is a potential biocontrol agent for the control of *S. lepidus* larvae in New Zealand pastures. Field trials established in 2005 showed a high variation of *B. bassiana* F418 population levels post-application between the different sites, time and treatments tested (Brownbridge *et al*., 2006). These findings highlighted the necessity of understanding the impact of abiotic and biotic factors on *B. bassiana* F418 population dynamics in soil (Brownbridge *et al*., 2006).

Many abiotic, biotic and pre-application factors can have an impact on the persistence of entomopathogenic fungi in soil such as moisture, temperature, soil texture, agricultural inputs, fungal formulation and interaction with soil microorganisms and plants (Jaronski, 2007). In general, persistence of entomopathogenic fungi is negatively affected by increasing soil moisture, temperature and peat content (Lingg & Donaldson, 1981; Studdert *et al*., 1990; Quintela *et al*., 1992; Vänninen *et al*., 2000). The effect of fertilisers, in particular nitrogen, gave divergent results amongst the different studies (Lingg & Donaldson, 1981; Rosin *et al*., 1996; Townsend *et al*., 2003).

The aim of this chapter was to understand how the persistence and virulence of *B. bassiana* F418 might be improved in soil under different abiotic and biotic soil conditions (soil moisture level, temperature, peat content, fertilisers and presence of host and non-host arthropods). The aim of this chapter was achieved by the following objectives (i) to test whether the effects of soil moisture, temperature and peat content on *B. bassiana* F418 persistence were similar to what was reported in the literature for other strains, (ii) to test the effect of nitrogen and superphosphate, two fertilisers commonly used on New Zealand pastures, on *B. bassiana* F418 persistence in soil and (iii) to determine the effect of the presence of a non-host arthropod on *B. bassiana* persistence in soil.

The effect of the different abiotic and biotic factors on *B. bassiana* F418 or F418 *gfp* tr1 persistence and virulence will be tested in a simplified microcosm of pasteurised soil without plant. If a factor modifies the population dynamics of *B. bassiana* F418 in pasteurised soil, it will then be tested in the more complex environment of non-sterile soil.

In Chapter 2, *B. bassiana* F418 *gfp* transformants were shown to be similar enough to the wild-type to be used as substitutes for *B. bassiana* F418. This conclusion will be further tested by using the transformants in pasteurised soil and comparing the population behaviour to that of the wild-type. This will determine if *B. bassiana* F418 *gfp* transformants can be used in subsequent experiments in non-sterile soil.

3.2 Methods

3.2.1 Soil collection and treatment

Templeton silt loam soil was obtained from an established pasture on the AgResearch Lincoln farm (43° 37' 44" S, 172° 28' 30" E). The Templeton silt loam texture is 57.5 % silt, 23.5 % clay and 19 % sand and contains 5.2 % organic matter (Appendix D). The pasture was a mix of perennial ryegrass (*Lolium perenne*, L., Poales, Poaceae), white clover (*Trifolium repens*, L., Fabales, Fabaceae) and red clover (*Trifolium pratense*, L., Fabales, Fabaceae) and was used for sheep grazing. The pasture receives 200 kg/ha of Sulphur Super 30 (S: 30.1, Ca: 16, P: 7, Ravensdown, NZ) per annum and 100 to 150 kg/ha of urea per annum. The top 10-15 cm was collected, sieved through a 4 mm mesh and plant debris and macro organisms such as worms were removed. Soil sample for pasteurisation was collected in May 2007 and oven-dried at 60 °C for 48 h and stored in plastic bags until use; the soil was then pasteurised for 48 h at 80 °C before experiments set up. Non-sterile soil was also collected from the same site in April 2008, sieved and stored at 4 °C in a sealed plastic bag until use within the same month. Soil samples of pasteurised and fresh soil were sent for analysis to NZlabs (Hamilton, NZ) and results are presented in Appendix D.

3.2.2 Fungal material

Beauveria bassiana F418 and its transformant F418 *gfp* tr1 were used for this study. The two strains were grown and harvested as described in section 2.2.1.2. *Beauveria bassiana* F418 and *B. bassiana* F418 *gfp* tr1 are referred to as F418 and F418 *gfp* tr1 respectively in this chapter.

3.2.3 Microcosms

Experiments were carried out in 250 mL screw cap containers (LabServ, NZ) filled with 150 g of dry pasteurised soil inoculated with 2×10^5 F418 conidia/g dry weight soil (DWS) and moistened to 22 % gravimetric water content (GWC) (=70 % field capacity (FC) Table 4.2) with sterile distilled water (SDW) (unless otherwise stated). The soil was thoroughly mixed with the water to the desired moisture level the day before the experiment was set up and kept at 20 °C ± 2. On the day the experiment was set up, the soil was mixed with the conidial suspension using a sterile spatula and the different treatments were applied as described in section 3.2.6. Containers were incubated at 15 °C (unless otherwise stated) in the dark using a randomized complete block design (RCBD). There were three replicate containers per treatment and one control per treatment which had 1 mL of 0.01 % Triton® X-100 (BDH, Poole, UK) instead of the conidial suspension.

3.2.4 Evaluation of persistence in soil

Persistence was monitored every week for four weeks, and then monthly up to five months of incubation (unless otherwise stated) using a soil dilution plating method based on Davet & Rouxel (2000). Five g of soil were sampled from each container using a 1 cm Ø soil core at three different collection points. The soil sample was mixed with 45 g of 0.01 % sterile distilled water agar (SDWA) (Appendix A) in a 250 mL screw cap container and this was treated as the 10^{-1} dilution. The containers were shaken on a vertical wrist shaker (Multi-wrist® shaker, Lab-Line instruments Inc., USA) for 10 min at maximum speed, and then left to stand 10 min before serial dilution. Serial dilutions from 10^{-2} to 5×10^{-4} were prepared and 100 µL of each dilution were spread onto Petri dishes of 10 % SDA+A. PDA+A (Appendix A) was used as a selective media for the first 4 weeks sampling before changing

for 10 % SDA+A. There were duplicate plates for each serial dilution. The plates were arranged in a RCBD design and incubated at 20 °C in the dark for 14 d before counting the CFU. Soil GWC was determined for each container after 24 h oven drying at 105 °C to allow calculation of CFU/g DWS. Persistence was expressed as the amount of CFU recovered/g DWS.

3.2.5 Evaluation of virulence

The virulence was assessed at each soil sampling by spreading 1 mL from the 10^{-1} soil dilution (section 3.2.4) onto a 90 mm Ø filter paper (LabServ, NZ) fitted inside a Petri dish with five 4-5th instar *T. molitor* larvae (Biosuppliers, Auckland) placed onto the filter paper. There were three replicate Petri dishes per soil container and the Petri dishes were arranged in a RCBD and incubated at 20 °C ± 2. Each block of plates was lying on a wet piece of paper and covered by a plastic cover to maintain high humidity during the experiment. The insect mortality was recorded every 2 d for three weeks. Virulence was expressed in LT_{50} in days after correction of the mortality data based on the mortality observed in the control using Abbott's formula (Abbott, 1925).

3.2.6 Evaluation of the effects of abiotic and biotic factors

3.2.6.1 Soil moisture content

The objective of this experiment was to assess the effects of different soil moisture levels on F418 persistence and virulence in pasteurised soil and determine if F418 persistence and virulence are better at some soil moisture level. The effect of soil moisture content on F418 persistence and virulence in pasteurised soil was determined by hydrating the pasteurised soil to 14, 31 and 22 % GWC as a control instead of only 22 % GWC described in section 3.2.3. Templeton silt loam field capacity is 31 mL H_2O/100 g of DWS (New Zealand Soil Bureau, 1968). Therefore the soils were brought to 45, 70 and 100 % FC (Table 4.2). The soil moistened to 14 % GWC was close to the wilting point. This experiment was set up in November 2007.

3.2.6.2 Soil temperature

The objective of this experiment was to assess the effects of different soil temperature on F418 persistence and virulence in pasteurised soil and determine if F418 persistence and virulence are better at some constant soil temperature. The effect of constant soil temperature on F418 persistence and virulence in pasteurised soil was determined by incubating the containers at 10, 20 and 15 °C as a control, instead of only 15 °C as described in section 3.2.3. This experiment was set up in November 2007.

3.2.6.3 Soil peat content

The objective of this experiment was to assess the effects of different soil peat content on F418 persistence and virulence in pasteurised soil and determine if F418 persistence and virulence are better with some soil peat content. The effect of soil peat content on F418 persistence and virulence in pasteurised soil was determined by using 120 g of pasteurised soil-peat mix containing 7.5 or 15 % of sphagnum peat moss (Hauraki gold, Yates, New Zealand) or no peat as a control, instead of 150 g of pasteurised soil only described in section 3.2.3.

Samples (500 mg) of soil with 0, 7.5 and 15 % peat were analysed to determine their C/N ratio by elemental analyser (Analytical Services Unit, Lincoln University, Lincoln, NZ). This experiment was set up in November 2007.

3.2.6.4 Host and non-host insects

3.2.6.4.1 Finding a non-host insect for F418

The objective of this experiment was to find a species of insect to which F418 is non-pathogenic for use as a non-host insect in future experiments. This objective was achieved by testing F418 against *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae), *Wiseana* spp. Viette (Lepidoptera: Hepialidae) and *C. zealandica* larvae. Details of the experiments are presented in Appendix E.

3.2.6.4.2 Effect of the presence of host and non-host insects on F418 in pasteurised soil

The objective of this experiment was to assess the effects of the presence of host, non-host or no insect on F418 persistence and virulence in pasteurised soil and this was determined by adding five 4-5th instar larvae of *T. molitor* (Biosupplier, Auckland, NZ) as a host insect or five 3rd instar of *C. zealandica* as a non-host insect or no insect as a control to the soil microcosm described in 3.2.3. *Costelytra zealandica* larvae were field collected (provided by R. Townsend, AgResearch, Lincoln, NZ) and quarantined in individual wells filled with soil, incubated at 10 °C for a week to check for potential disease. Healthy *C. zealandica* larvae were retrieved from the wells, sprayed with water to remove any soil particles and left to dry on filter paper for a few minutes before use. To ensure the well-being of the larvae, 10 cubes (approximately 5 × 5 mm) of carrot (non-sterile) were added in each container with *C. zealandica* larvae and 1 g of non-sterile cereal bran was added to each container with *T. molitor* larvae. The food was added at the beginning of the experiment and during the second week sampling. Containers with *T. molitor* larvae were turned over gently every week to ensure the larvae crawled through the soil, since the larvae would otherwise stay at the soil surface. *Tenebrio molitor* larvae mortality was monitored at each soil sampling. This experiment was set up in November 2007.

3.2.6.5 Fertilisers

3.2.6.5.1 Effect of fertilisers on F418 in pasteurised soil (November 2007)

The objective of this experiment was to assess the effects of nitrogen and superphosphate fertilisers on F418 persistence and virulence in pasteurised soil and determine if F418 persistence and virulence are better with some type of fertilisers. The effect of fertiliser on F418 persistence and virulence in pasteurised soil was determined by adding 128 mg of urea (46 N, n-rich™, Ballance™, NZ) or 320 mg of single superphosphate (P 9: S 10.5: Ca 22, superten™, Ballance™, NZ), or both urea and single superphosphate, or no fertiliser as a control to 150 g of pasteurised soil microcosm described in 3.2.3. The urea and single superphosphate were prepared as a suspension in SDW with 128 mg n-rich™/mL and 32 mg superten™/mL. The n-rich™ application rate used was 400 kg/ha whereas the recommended application rate is a maximum of 120 kg/ha on dairy pastures in spring. The superten™ application rate used was 1000 kg/ha whereas the recommended application

rate is a maximum of 450 kg/ha on dairy pasture per year. The rate of urea and single superphosphate was two to three times the recommended rate to induce some exaggerated effect of fertilisers. This experiment was set up in November 2007.

3.2.6.5.2 Effect of fertilisers on F418 and F418 *gfp* tr1 persistence in pasteurised and non-sterile soil (April 2008)

The objectives of this experiment were to assess the effects of nitrogen and superphosphate fertilisers on F418 and F418 *gfp* tr1 persistence in pasteurised and/or non-sterile soil, and determine if F418 persistence was similar to F418 *gfp* tr1 in pasteurised soil under similar fertiliser treatments, and finally to determine if F418 *gfp* tr1 persistence in non-sterile soil was the same than in pasteurised soil under similar fertiliser treatments.

The effect of type of fertilisers on F418 and F418 *gfp* tr1 persistence and virulence in soil was determined by adding 32 mg of urea (46 N, n-rich™, Ballance™, NZ) or 85 mg of single superphosphate (P 9: S 10.5: Ca 22, superten™, Ballance™, NZ) or both urea and single superphosphate or no fertiliser as a control to 120 g of dry pasteurised soil and 142.85 g of non-sterile soil (at 19 % GWC therefore equivalent to 120 g of dry soil). The urea and single superphosphate were prepared as suspension in SDW with 10.65 mg n-rich™/mL and 16.90 mg superten™/mL. The n-rich™ application rate used was 100 kg/ha and the superten™ application rate used was 270 kg/ha which are both under the maximum application rate recommended. The fertiliser treatments were applied to pasteurised soil with F418, pasteurised soil with F418 *gfp* tr1 and non-sterile soil with F418 *gfp* tr1. All soils were moistened to 28 % GWC instead of the 22 % GWC described in section 3.2.3. The rest of the experiment was set up as described in section 3.2.3 apart from that the containers were incubated using a split plot design and both soils were kept at 4 °C for 24 h before the experiment was set up. The evaluation of persistence was set up as described in section 3.2.4 apart from that the serial dilutions were spread onto Petri dishes of 10 % SDA+A+Hyg when F418 *gfp* tr1 was used and the dishes were incubated using a split plot design. PDA+A plates were not used in this experiment. The virulence was assessed by using the 10^{-2} soil dilution instead of 10^{-1} described in section 3.2.5 to allow a better differentiation in virulence. This experiment was set up in April 2008.

3.2.6.6 Other factors

The persistence of F418 in pasteurised soil was also assessed with conidia grown on different media, with conidia from 10 % SDA medium incubated for different length of time, with ungerminated and pre-germinated conidia, and in fresh and used pasteurised soil. The results of these experiments are presented in Appendix G as only the factors that had the greatest effects were chosen to appear inside the experimental chapter.

3.2.7 Statistical analysis

All CFU recovery data were $\log_{10}$ transformed to achieve a normal distribution essential to the use of ANOVA (Olsen, 2003). A split plot ANOVA was carried out, with treatment factors being the treatments (i.e. soil moisture, temperature, peat content, insects, fertilisers, soil type, fungus) and time, with a linear polynomial contrast specified where appropriate. Each container was treated as a main plot so an ANOVA could be used. Virulence was expressed as the LT_{50} (days) determined by probit analysis after correction of the mortality data based on the control group mortality using Abbott's formula (Abbott, 1925) when necessary. LT_{50} values obtained after probit analysis were $\log_{10}$ transformed and compared by general ANOVA with factorial treatments structures and interactions. Linear regression analyses were carried out to test the correlation between the virulence (LT_{50}) and F418/F418 *gfp* tr1 populations' levels. Significance was evaluated at $P < 0.05$ for all analyses and mean separation was executed by the unprotected LSD test. All data analyses were performed using the statistical software Genstat version 12.2 (VSN International). All statistical results tables are presented in Appendix H.

3.3 Results

3.3.1 Effect of moisture content on F418 persistence and virulence in pasteurised soil

The effect of soil moisture content on the persistence of F418 in pasteurised soil was assessed over 21.5 weeks (Fig. 3.1). F418 populations increased significantly in all soil moisture contents over the first two weeks compared to the initial inoculum level (INIL) which had an average of 1.36×10^5 CFU/g DWS (=5.132 in $\log_{10}$). However, the increase was significantly higher in 14 % GWC soil than in the two wetter soils at 1 and 2 weeks. After 2 weeks, F418 populations continually decreased in soil at 31 % GWC and were significantly lower than the two other treatments between 4 and 21.5 weeks. F418 populations were also significantly lower than the INIL at 12, 17 and 21.5 weeks and was only 14 % of the INIL at 21.5 weeks with 2.12×10^4 CFU/g DWS (=4.326 in $\log_{10}$). Whereas, F418 populations in soil at 14 and 22 % GWC were maintained at a level significantly higher than the INIL between 4 and 21.5 weeks with 533 % and 212 % of the INIL left at 21.5 weeks in soil at 14 and 22 % GWC, respectively. F418 populations were significantly different between each treatment at 17 and 21.5 weeks with the population being significantly higher in soil at 14 % GWC, followed by soil at 22 % GWC and the lowest being in soil at 31 % GWC.

PDA+A plates were used between 0 and 4 weeks and contamination by other microorganisms could be observed between 1 and 4 weeks. Plates with soil dilutions from 31 % GWC soil were more heavily contaminated than the plates with dilutions from the drier soils. Soil dilutions were plated onto 10 % SDA+A plates between 9 and 21.5 weeks and little contamination occurred on this more selective media.

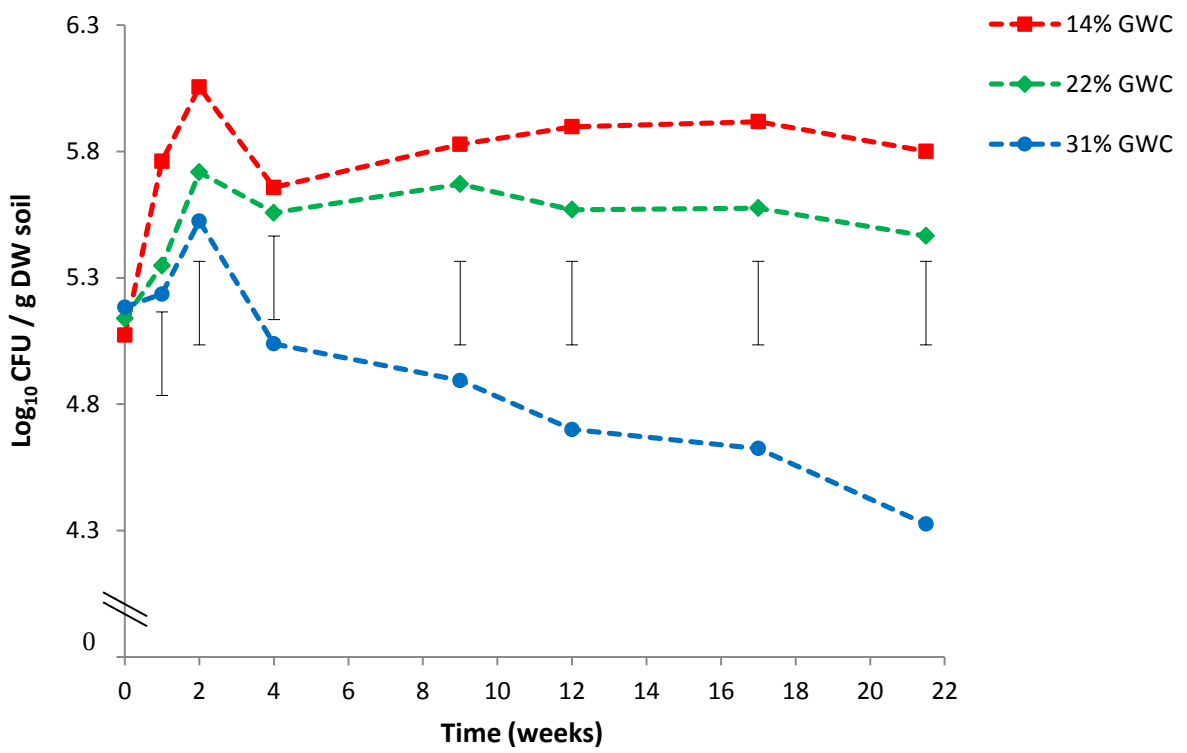

Figure 3.1 F418 populations recovered (log_{10} CFU/g DWS) in pasteurised soil at 14, 22 and 31 % GWC over 21.5 weeks at 15 °C in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

The effect of soil moisture content on the virulence of F418 in pasteurised soil was assessed over 21.5 weeks (Fig. 3.2). The virulence increased (LT_{50} decreased) for all soil treatments during the first 2 weeks where the F418 populations increased in all soil treatments during that time (Fig 3.1). Virulence of F418 in soil with 31 % GWC was significantly lower (LT_{50} higher) than with the two drier soils from 9 to 21.5 weeks (LSD = 0.066), while F418 population recovered from soil at 31 % GWC was also significantly lower compared to the two drier soils (Fig 3.1). At 22 weeks, virulence was significantly different between all soil treatments with the virulence being significantly higher in soil at 14 % GWC, followed by soil at 22 % GWC and the lowest being in soil at 31 % GWC.

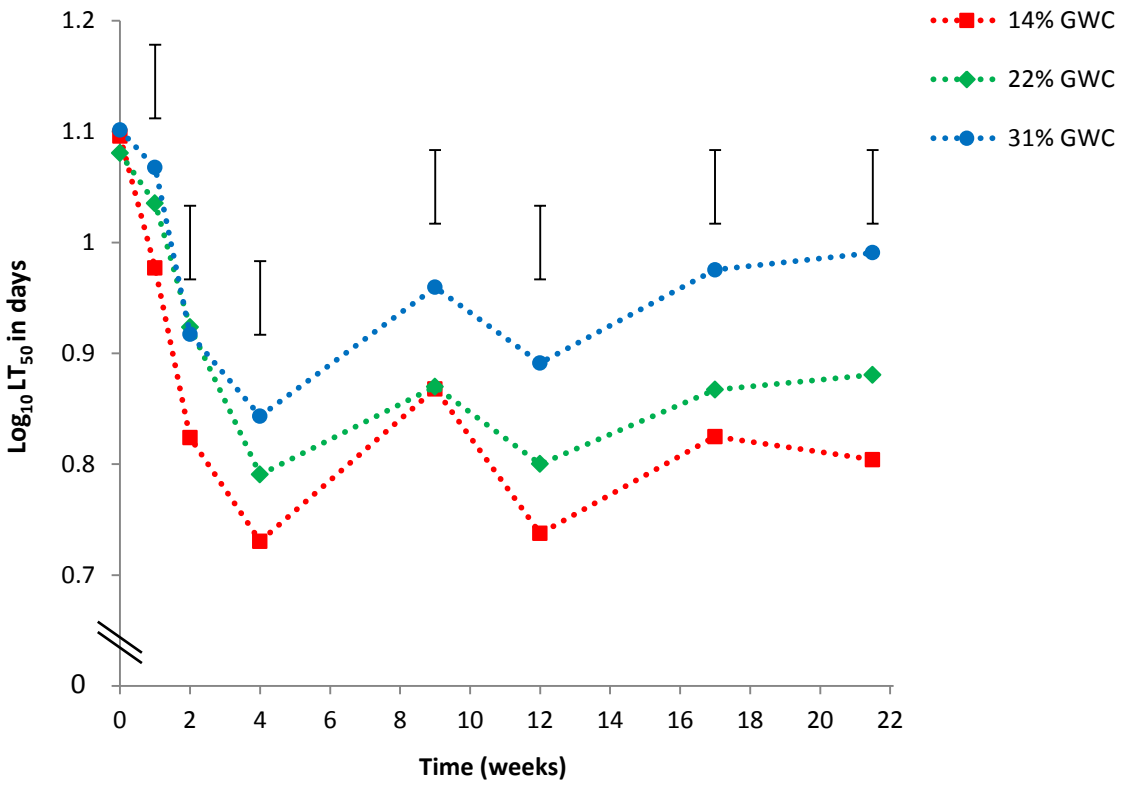

Figure 3.2 Virulence (log_{10} of LT_{50} in days) against *T. molitor* larvae of F418 in soil at 14, 22 and 31 % GWC over 21.5 weeks. Vertical bars are LSD ($P < 0.05$).

The virulence values (LT_{50} in days) were significantly correlated to the F418 populations values (log_{10} CFU/g DWS) with a P value of < 0.001 which means that the virulence depended on the F418 populations level with the virulence being higher (LT_{50} lower) when the F418 populations were higher in soil (Fig 3.3).

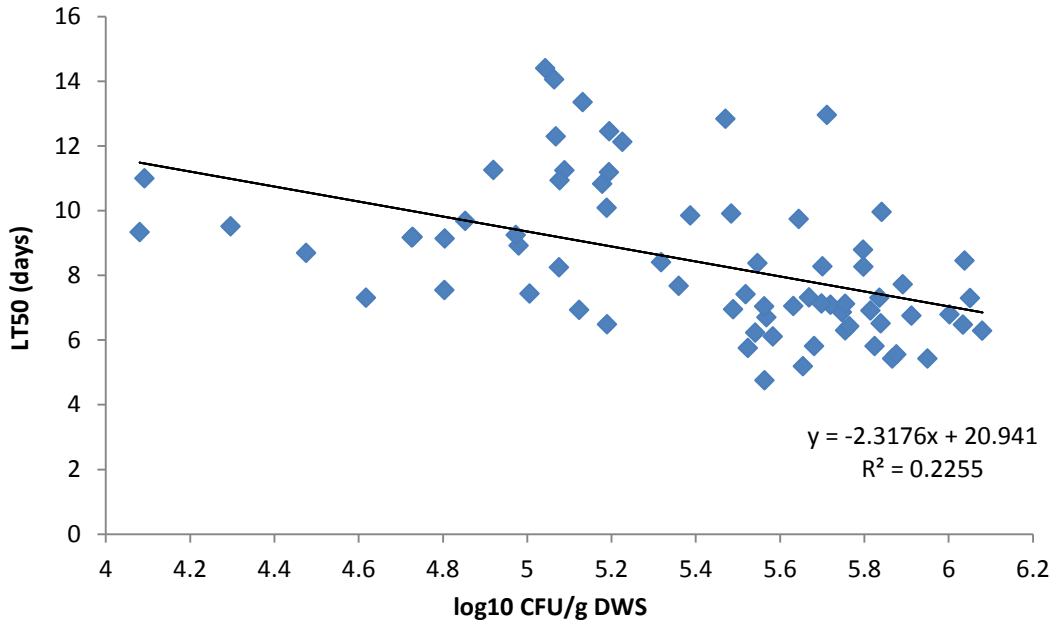

Figure 3.3 Linear correlation between virulence (LT_{50} in days) against *T. molitor* larvae and log_{10} of CFU/g DWS of F418 in pasteurised soil at 14, 22 and 31 % GWC over 21.5 weeks at 15 °C ($P < 0.001$).

3.3.2 Effect of soil temperature on F418 persistence in pasteurised soil

The effect of constant soil temperature on the persistence and virulence of F418 in pasteurised soil was assessed over 21.5 weeks (Fig. 3.4 and Appendix F). F418 populations increased significantly at all soil temperatures over the first 4 to 9 weeks compared to the INIL which had an average of 1.41×10^5 CFU/g DWS (=5.148 in log_{10}). However, populations increase was delayed in soil at 10 °C as the populations were not significantly different from the INIL at 1 and 2 weeks whereas the populations at 15 and 20 °C were significantly higher than the INIL. F418 populations at 10 and 15 °C were maintained at a significantly high level (compared to INIL) between 2 and 21.5 weeks and were 214 % and 268 % the INIL at 21.5 weeks at 10 °C (with 3.33×10^5 CFU/g DWS = 5.523 in log_{10}) and 15 °C (with 3.81×10^5 CFU/g DWS = 5.581 in log_{10}) respectively. F418 populations at 20 °C decreased continually between 9 and 21.5 weeks until it was not different from the INIL at 21.5 weeks and were significantly lower than populations at 10 and 15 °C at the last sampling.

PDA+A plates were used between 0 and 4 weeks and some contamination by other microorganisms could be observed between 1 and 4 weeks, although no qualitative

difference of contamination could be seen between the different soil temperature treatments. Soil dilutions were plated onto 10 % SDA+A plates between 9 and 21.5 weeks and little contamination occurred on this more selective media.

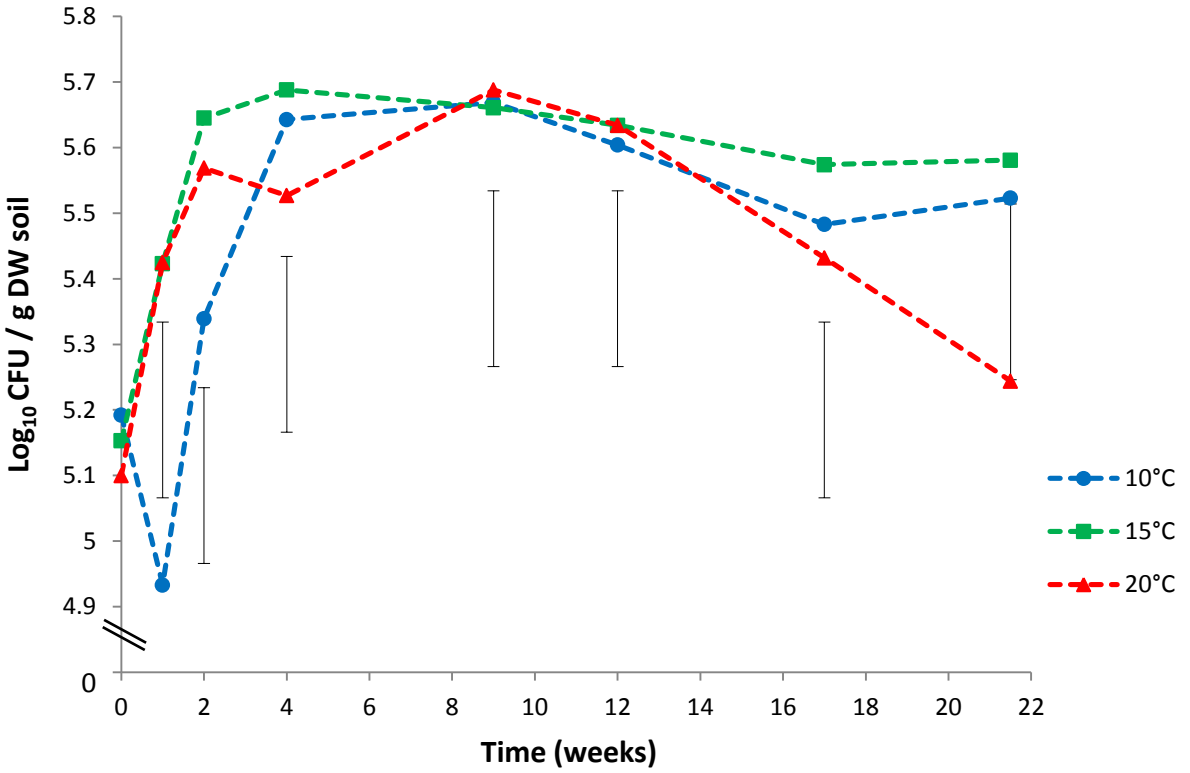

Figure 3.4 F418 populations recovered (log_{10} CFU/g DWS) in pasteurised soil at 22 % GWC at 10, 15 and 20 °C over 21.5 weeks in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

.

3.3.3 Effect of peat content on F418 persistence in pasteurised soil

The effect of soil peat content on the persistence and virulence of F418 in pasteurised soil was assessed over 21.5 weeks (Fig. 3.5 and Appendix F). F418 populations increased significantly in all soil peat contents over the first two weeks compared to the INIL which had an average of 1.36×10^5 CFU/g DWS (=5.133 in log_{10}). However, F418 populations were significantly different between all treatments at 2 weeks, with the highest populations being in soil with 7.5 % peat, followed by soil with 15 % peat and the lowest populations were in soil without peat. F418 populations were maintained significantly higher than the INIL in soil with 7.5 % peat between 1 and 21.5 weeks and the populations were 251 % the INIL (3.44×10^5 CFU/g DWS =5.536 in log_{10}) at 21.5 weeks. F418 populations in soil without peat were

maintained significantly higher than the INIL between 2 and 12 weeks followed by a decrease back to the INIL at 17 and 21.5 weeks. F418 populations in soil with 15 % peat decreased continually between 2 and 21.5 weeks and were not significantly different from the INIL between 9 and 17 weeks. It was significantly lower than the INIL at 21.5 weeks with 1.88×10^4 CFU/g DWS (=4.274 in $\log_{10}$) which is only 15 % of the INIL. F418 populations were significantly different between all the treatments between 12 and 21.5 weeks with the highest populations being in soil with 7.5 % peat, followed by soil without peat and the lowest populations in soil with 15 % peat.

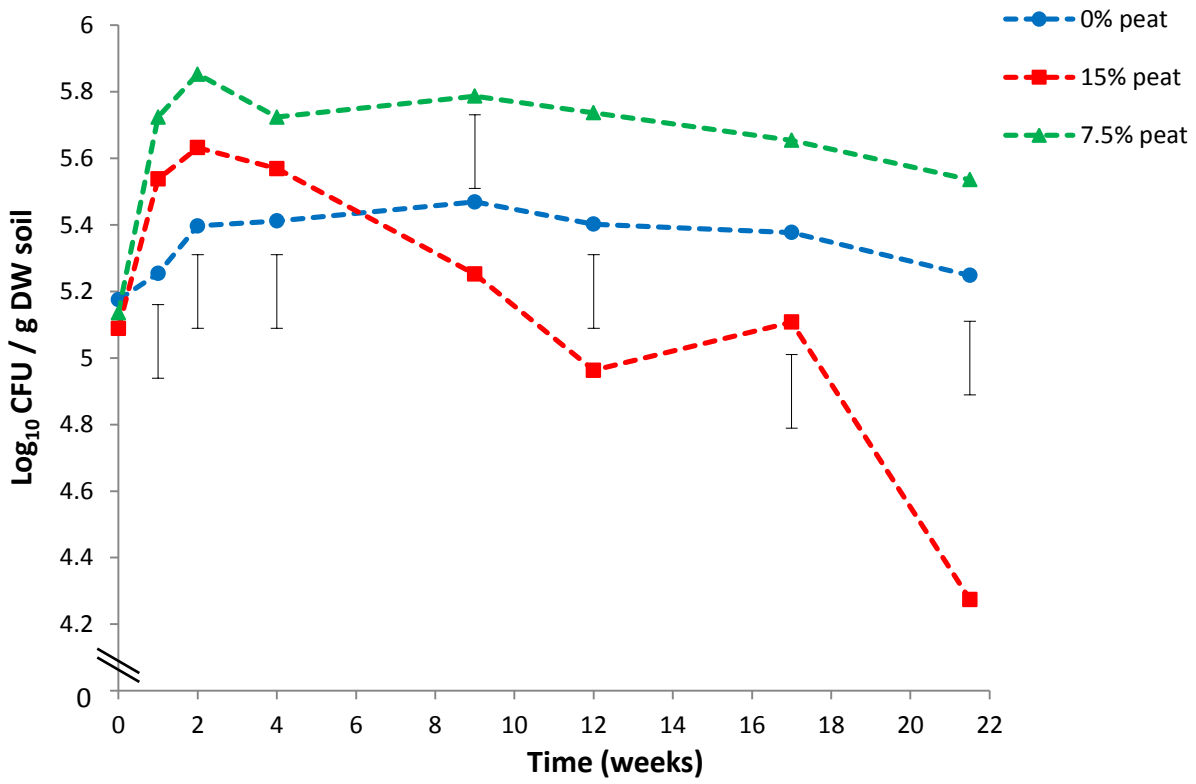

Figure 3.5 F418 populations recovered ($\log_{10}$ CFU/g DWS) in pasteurised soil at 15 °C and 22 % GWC with 0, 7.5 and 15 % peat over 21.5 weeks in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

Soil pH was measured at 29 weeks and the pH of soil without peat was 5.72, soil with 7.5 % peat was 5.65 and soil with 15 % peat was 5.05.

Soils with different peat content were analysed for their nitrogen and carbon content (Table 3.1). Soil without peat had a C/N ratio of 11.3, soil with 7.5 % peat had a C/N ratio of 20 and soil with 15 % peat had a C/N ratio of 24.9.

Table 3.1 Nitrogen, carbon and C/N ratio of the soil with different peat content

	% Nitrogen	% Carbon	C/N ratio
Soil without peat	0.26	2.91	11.3
Soil + 7.5 % peat	0.31	6.24	20.0
Soil + 15 % peat	0.37	9.23	24.9

3.3.4 Effect of the presence of host or non-host insects on F418 persistence in pasteurised soil

The effect of presence of insects on the persistence and virulence of F418 in pasteurised soil was assessed during 17 weeks (Fig. 3.6 and Appendix F). F418 populations in soil with host insect was significantly lower than the INIL of 3.20×10^5 CFU/g DWS (=5.505 in $\log_{10}$) at 2 and 4 weeks (LSD = 0.497). It was observed that the cereal bran for feeding the *T. molitor* larvae was mouldy and all the PDA+A plates used for the recovery of F418 between 1 and 4 weeks were heavily contaminated by a fungus which was either an *Aspergillus* sp. P. Micheli ex Haller (Ascomycota: Eurotiales) or *Penicillium* sp. Therefore, the low F418 populations observed at 2 and 4 weeks were likely to be either an artefact due to the contamination of the PDA+A plates by a food mould which would have masked the real number of F418 CFU on the plates or a real decrease of F418 populations because of an antagonism with the food mould. F418 populations in soil with host insects increased up to the INIL between 9 and 17 weeks and were 290 % the INIL at 17 weeks with 9.27×10^5 CFU/g DWS (= 5.967 in $\log_{10}$) (LSD = 0.497). All *T. molitor* larvae in soil with F418 died within 4 weeks but did not show any conidiation until the 9, 12 and 17 weeks sampling.

F418 populations in soil with non-host and without insects increased over the first 2 to 4 weeks although it was not significantly different from the INIL. F418 populations in soil with non-host and without insects were not significantly different from the INIL between 1 and 17 weeks and were 75 and 278 % of the INIL at 17 weeks, respectively. F418 populations in soil with non-host insects were significantly lower than in soils with host insects and without insects at 17 weeks.

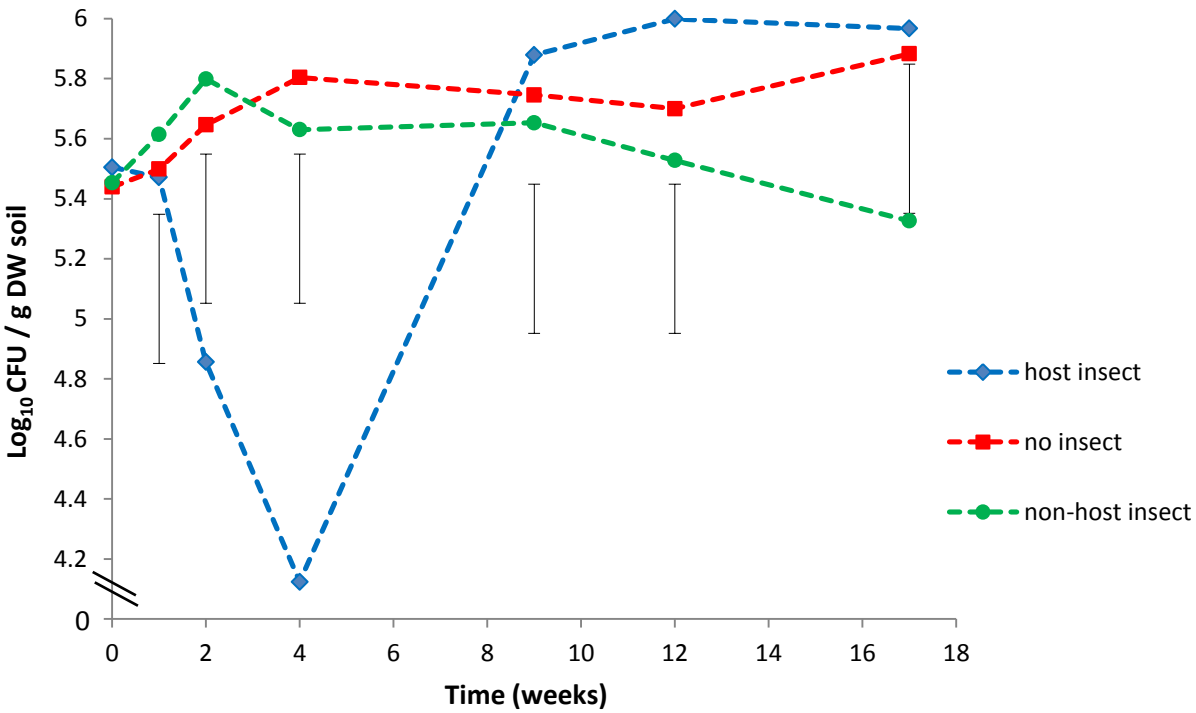

Figure 3.6 F418 populations recovered (log_{10} CFU/g DWS) in pasteurised soil at 15 °C and 22 % GWC with host, non-host insect or no insect over 17 weeks in laboratory microcosms. Vertical bars are LSD (P < 0.05).

3.3.5 Effect of fertilisers on F418 in pasteurised soil

3.3.5.1 November 2007 experimental results

The effect of fertilisers on the persistence and virulence of F418 in pasteurised soil was assessed over 29 weeks (Fig. 3.7 and Appendix F). Soil amended with single superphosphate will be referred as soil with P, soil amended with urea will be referred as soil with N, soil amended with both single superphosphate and urea will be referred as soil with N+P.

F418 populations increased significantly in all soil treatments over the first two weeks compared to the INIL which had an average of 2.06×10^5 CFU/g DWS (=5.314 in log_{10}) but in soil with N + P the populations increased significantly only at 9 weeks. F418 populations in soil with P were maintained significantly higher than the INIL between 1 and 29 weeks and was 234 % the INIL at 29 week with 4.10×10^5 CFU/g DWS (=5.613 in log_{10}). F418

populations in soil without fertiliser were maintained significantly higher than the INIL between 2 and 17 weeks followed by a decrease resulting in no significant difference to the INIL at 29 weeks.

F418 populations in soil with N were not significantly different from the INIL between 4 to 17 weeks followed by a decrease significantly lower than the INIL at 29 weeks, with only 11 % of the INIL left (2.28×10^4 CFU/g DWS = 4.358 in $\log_{10}$). Similarly, F418 populations in soil with N+P were not significantly different from the INIL at 1, 2, 4, 12 and 17 weeks followed by a decrease significantly lower than the INIL at 29 weeks with 36 % left of the INIL (8.09×10^4 CFU/g DWS = 4.908 in $\log_{10}$).

F418 populations in soil with P were significantly higher than in soil with N and soil with N+P at 4, 9, 17 and 29 weeks. At 29 weeks, F418 populations in soil with P and soil without fertiliser were significantly higher than in soil with N+P and populations in soil with N+P was significantly higher than in soil with N. The level of F418 population in soil with N+P was intermediate to the levels of population in soil with P and in soil with N from 8 to 29 weeks.

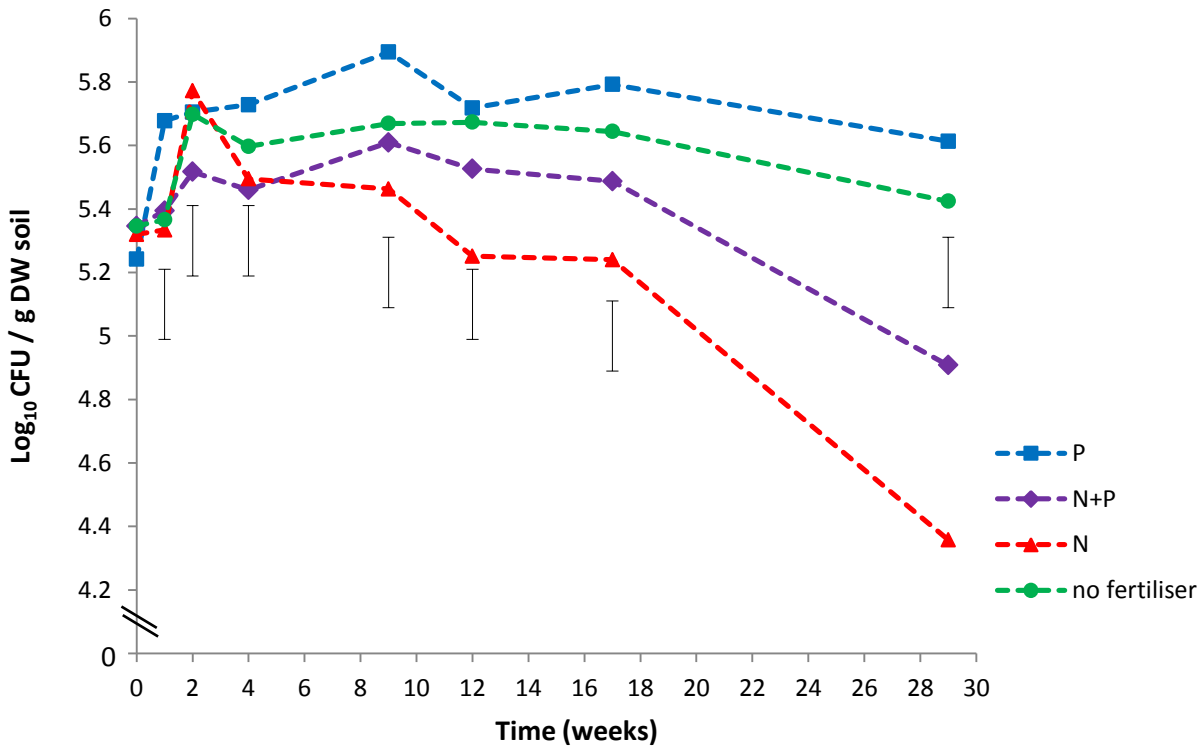

Figure 3.7 F418 populations recovered ($\log_{10}$ CFU/g DWS) in pasteurised soil at 22 % GWC and 15 °C amended with superphosphate (P), urea (N), both fertliser (N+P) or no fertiliser over 29 weeks set up in November 2007 in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

3.3.5.2 April 2008 experimental results

The effect of fertilisers on the persistence of F418 in pasteurised soil was assessed over 13 weeks (Fig. 3.8). F418 populations decreased in all soil treatments and were all significantly lower than the average INIL of 1.53×10^5 CFU/g DWS (= 5.185 in $\log_{10}$) at 13 weeks, although there were some significant differences between the treatments.

F418 populations in soil with P were significantly higher than all the other treatments at 8 weeks and were significantly higher than in soil with N and N+P at 13 weeks. F418 populations in soil with P were 42 % of the INIL at 13 weeks with 6.59×10^4 CFU/g DWS (= 4.819 in $\log_{10}$). F418 populations in soil without fertiliser (4.13×10^4 CFU/g DWS = 4.645 in $\log_{10}$) were significantly higher than in soil with N (1.51×10^4 CFU/g DWS = 4.187 in $\log_{10}$) and soil with N+P (2.11×10^4 CFU/g DWS = 4.328 in $\log_{10}$) (LSD = 0.291) at 13 weeks and were 29 % of the INIL at 13 weeks. F418 populations in soil with N+P were 15 % of the INIL and F418 populations in soil with N were 9 % of the INIL at 13 weeks. The level of F418 populations in soil with N+P was intermediate to the levels of populations in soil with P and in soil with N at 8 and 13 weeks.

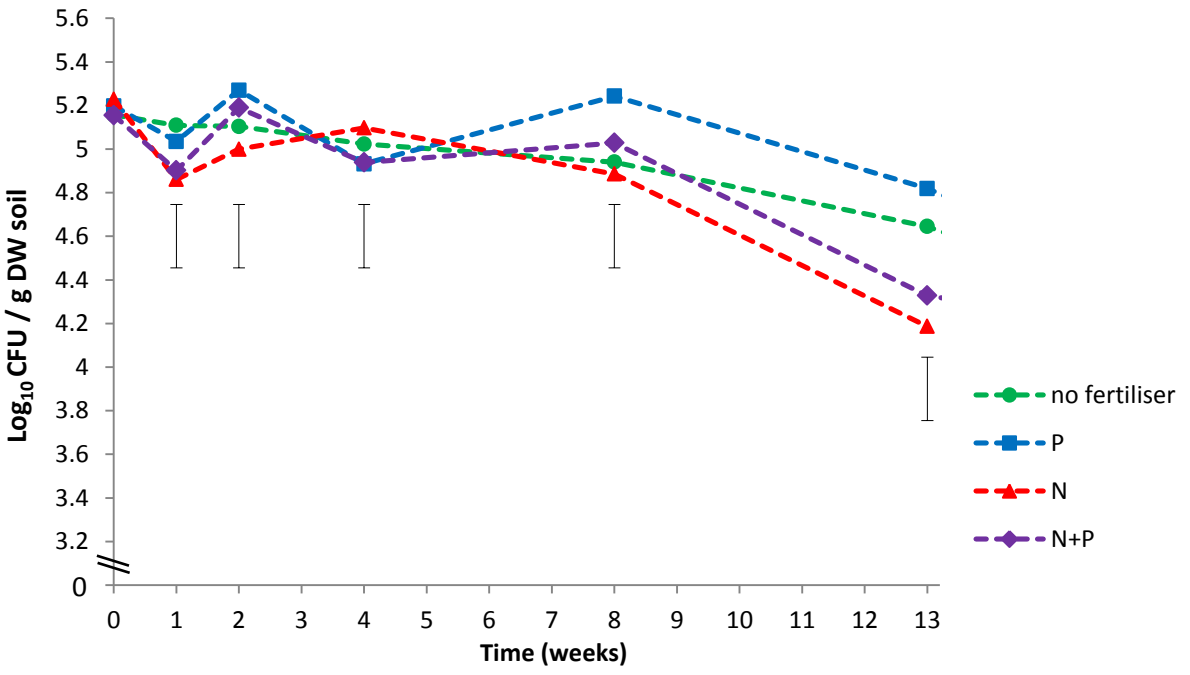

Figure 3.8 F418 populations recovered ($\log_{10}$ CFU/g DWS) in pasteurised soil at 22 % GWC and 15 °C amended with superphosphate (P), urea (N), both fertliser (N + P) or no fertiliser over 13 weeks set up in April 2008 in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

3.3.6 Effect of fertilisers on F418 *gfp* tr1 in pasteurised soil

The effect of fertilisers on the persistence of F418 *gfp* tr1 in pasteurised soil was assessed over 22 weeks (Fig. 3.9). F418 *gfp* tr1 populations decreased in all soil treatments and were all significantly lower than the average INIL of 1.77×10^5 CFU/g DWS (= 5.248 in $\log_{10}$) at 22 weeks, although there were some significant differences between the treatments.

F418 *gfp* tr1 populations in soil with P (7.48×10^3 CFU/g DWS = 3.874 in $\log_{10}$) were significantly higher than in all the other treatments at 22 weeks and were 4.17 % of the INIL. F418 *gfp* tr1 populations in soil without fertiliser (3.08×10^3 CFU/g DWS = 3.489 in $\log_{10}$) were significantly higher than in soil with N (1.21×10^3 CFU/g DWS = 3.084 in $\log_{10}$) (LSD = 0.291) at 22 weeks and were 1.76 % of the INIL. F418 *gfp* tr1 populations in soil with N+P were not significantly different from populations in soil with N at 22 weeks. F418 *gfp* tr1 populations in soil with N+P were 0.96 % of the INIL at 22 weeks and F418 *gfp* tr1 populations in soil with N were 0.84 % of the INIL at 22 weeks. The level of F418 *gfp* tr1 populations in soil with N+P was intermediate to the levels of populations in soil with P and in soil with N at 8, 13 and 22 weeks.

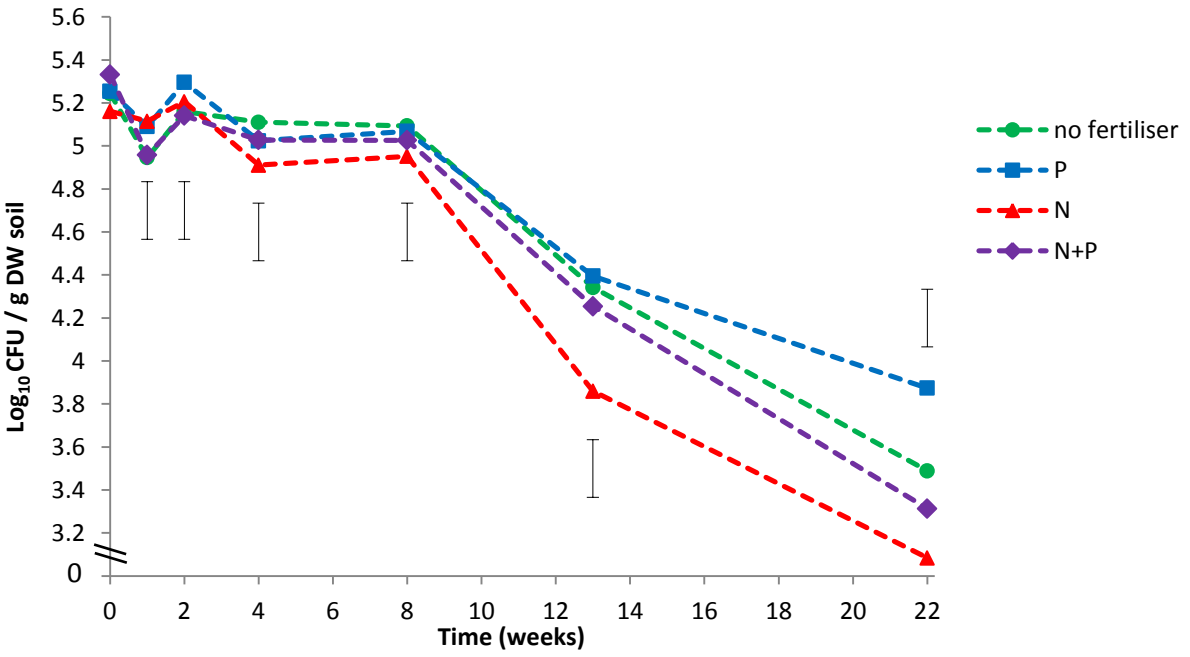

Figure 3.9 F418 *gfp* tr1 populations recovered ($\log_{10}$ CFU/g DWS) in pasteurised soil at 22 % GWC and 15 °C amended with superphosphate (P), urea (N), both fertliser (N + P) or no fertiliser over 22 weeks in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

3.3.7 Effect of fertilisers on F418 *gfp* tr1 in non-sterile soil

The effect of fertilisers on the persistence of F418 *gfp* tr1 in non-sterile soil was assessed over 22 weeks (Fig. 3.10). F418 *gfp* tr1 populations in soil with all the different treatments were not significantly different from the INIL at 1, 2, 4, 13 and 22 weeks. F418 *gfp* tr1 populations in soil with P and without fertiliser were significantly higher than the INIL at 8 weeks. F418 *gfp* tr1 populations in soil without fertiliser (8.41×10^4 CFU/g DWS = 4.925 in $\log_{10}$) were significantly lower than in soil with N+P (1.75×10^5 CFU/g DWS = 5.242 in $\log_{10}$) (LSD = 0.291) at 22 weeks. At 22 weeks, F418 *gfp* tr1 populations were 90 % of the INIL in soil with N+P, 85 % of the INIL in soil with P, 76 % of the INIL in soil with N and 64 % of the INIL in soil without fertiliser.

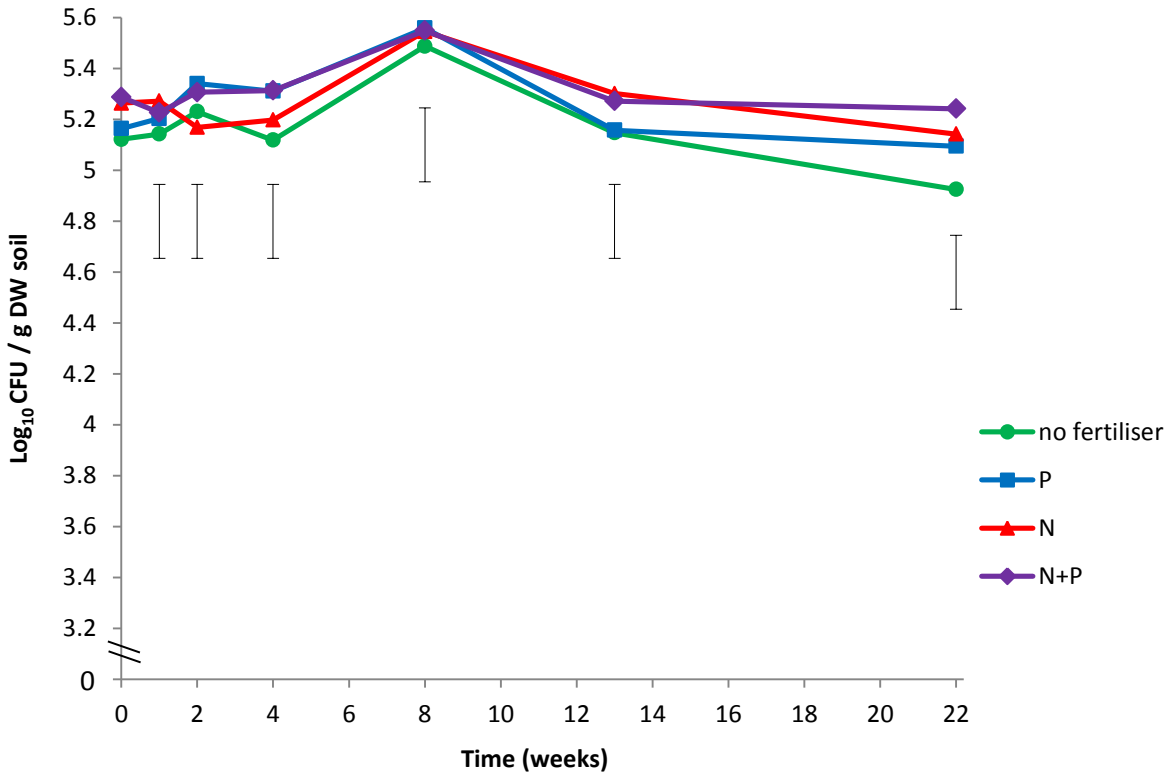

Figure 3.10 F418 *gfp* tr1 populations recovered ($\log_{10}$ CFU/g DWS) in non-sterile soil amended with superphosphate (P), urea (N), both fertliser (N + P) or no fertiliser over 22 weeks at 22 % GWC and 15 °C in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

3.4 Discussion

3.4.1 F418 and F418 *gfp* tr1 population dynamics in pasteurised soil

A F418 population increase was observed during the first two to four weeks for all experiments in pasteurised soil set up in November 2007. *Beauveria bassiana* population increase in sterile soil has been observed previously by Lingg & Donaldson (1981), Quintela *et al*. (1992) and Pereira *et al.* (1993). Lingg & Donaldson (1981) observed mycelia growth of *B. bassiana* on the surface of the sterile soil, while Pereira *et al.* (1993) also observed conidiation of *B. bassiana* on the sterile soil surface. Walstad *et al.* (1970), Lingg & Donaldson (1981) and Pereira *et al.* (1993) noted absence of germination and growth of *B. bassiana* in non-sterile soil and concluded that fungistasis prevented *B. bassiana* germination in non-sterile soil by either nutrient competition or inhibition by antagonists. Pasteurised soil contained a limited microbial population, therefore the fungistasis is probably limited at the beginning of the experiment, at least, and the absence of fungistasis could explain the increase of F418 populations during the first few weeks. There was no F418 populations increase past this initial growth which may be due to a lack of nutrient availability following the initial increase. No increase of F418 populations was observed during the first four weeks in pasteurised soil in the fertiliser experiments set up in April 2008.

Pasteurised soil for experiments set up in November 2007 was stored at 20 °C ± 2 for 24 h prior setting up the experiment whereas pasteurised soil for experiments set up in April 2008 was stored at 4 °C, all soils were thereafter incubated at 15 °C. F418 population in pasteurised soil with F418 grown on different media set up in February 2008 (Appendix G) had a maximum increase average of 150 fold the INIL whereas F418 populations in experiments set up in November 2007 had a maximum increase ranging from 2.5 to 5.2 fold the INIL. The pasteurised soil used for the experiment with different growing media (Appendix G) had been removed from the 80 °C oven (for pasteurisation) a short time before setting up the experiment, therefore the soil was warmer than 20 °C. Thus, the soil temperature during inoculation appeared to have a strong impact on the population persistence but this was not investigated further. Faria *et al.* (2009) studied the germination rate of *B. bassiana* conidia after being exposed at different imbibition temperature. *Beauveria bassiana* conidia were stored for 3 d at 25 °C in jars with different salts to give

them different water activity, then quickly added to vials of water and Lutensol at 0.5, 15, 25 and 33 °C, vortexed and maintained for 2 min at these temperatures before doing a germination test. After incubation at 25 °C for 48 h, the germination rate of conidia imbibed at 0.5 °C was significantly lower than for conidia imbibed at warmer temperatures at all water activities. Faria *et al.* (2009) showed that the temperature at which *B. bassiana* conidia were imbibed for just 2 min, had an important impact of the germination rate and this finding might explain in part the persistence results observed in the present study when F418 was inoculated in soil at different temperature.

3.4.2 Impact of abiotic and biotic factors on F418 and F418 *gfp* tr1 persistence

3.4.2.1 Impact of soil moisture on F418 persistence

F418 persistence in pasteurised soil was negatively influenced by the level of moisture of the soil between 14 and 31 % GWC. This observation is similar to that of Lingg & Donaldson (1981), Studdert *et al*. (1990) and Quintela *et al*. (1992). Lingg & Donaldson (1981) found that *B*. *bassiana* M-4 conidia survival in autoclaved soil decreased as soil moisture content increased from 25 to 75 % saturation. Studdert *et al*. (1990) also showed that *B*. *bassiana* conidia half-lives (ABG-6178 and IL-116 strains) were higher in air dried soil when water potential was at -15 bar than at 0 bar (saturation), although they used non-sterile soil in their study. Quintela *et al*. (1992) observed that the persistence of *B. bassiana* CP5 was higher in autoclaved soil at 25 than at 75 % saturation.

Both Lingg & Donaldson (1981) and Studdert *et al.* (1990) suggested that *B. bassiana* conidial survival in wetter non-sterile soil was affected by the soil microbial population (such as antagonist bacteria) which is more active in wetter soil. Plates with pasteurised soil dilution from 31 % GWC were heavily contaminated by other microorganisms compared to soil dilution plating from drier soils when PDA+A was used. The qualitative difference in contamination showed that there might be more microorganisms, and possibly more antagonists present in the soil at 31 % GWC. Lingg & Donaldson (1981) also suggested that a soil atmosphere reduced in O_2 and enriched in CO_2 could be responsible for the reduced conidial survival in wetter soil. However, the 31 % GWC soil is equivalent to 100 % field capacity, therefore the soil was not saturated in water and there would have been a reasonable amount of available O_2.

It is likely that the difference in F418 persistence observed in pasteurised soil with different levels of moisture content was due to a change in soil microorganisms present in the soils at different moisture levels. This hypothesis could be tested by studying the soil microbial communities present in the soils with different soil moisture.

3.4.2.2 Impact of soil temperature on F418 persistence

F418 persistence in pasteurised soil decreased at high soil temperature (20 °C) compared to cooler temperature (10 and 15 °C). Similarly, Lingg & Donaldson (1981) found that *B*. *bassiana* M-4 and CLB-2 conidial survival was greater at 10 °C than 25 °C in an autoclaved silt loam soil. Studdert *et al*. (1990) showed an inverse relationship between persistence and soil temperature over a range from 10 to 40 °C in two types of non-sterile soil and hypothesised that the loss of conidia survival with increasing temperature may be due to a higher activity of microorganisms degrading conidia with the increasing soil temperature.

Temperature alone is known to have a direct impact on *B. bassiana* persistence in shelf life studies. Hong *et al.* (2001) found that the longevity of *B*. *bassiana* conidia was negatively correlated with increasing storage temperatures from 10 to 50 °C. Sandhu *et al*. (1993) reported similar findings. F418 populations increase were delayed by one week in soil at 10 °C which is likely due to the slower germination rate and colony growth observed at lower temperature compared to higher temperature (section 2.3.1, Fargues *et al.*, 1997; Studdert & Kaya, 1990).

F418 persistence in pasteurised soil is affected by high soil temperature and this could be due to either a direct impact of the temperature or an indirect impact of the temperature on the development of antagonists in soil.

3.4.2.3 Impact of peat content on F418 persistence

F418 populations in pasteurised soil were influenced by the peat content of the soil but the correlation was not linear. F418 persistence in soil with 15 % peat was low after 22 weeks. This finding is similar to Studdert *et al.* (1991) who observed that *B. bassiana* conidia survival was low in a peat soil compared to a silt loam soil and suggested that the peat soil which had a high content in organic matter (62 %) could have a higher population of

microorganisms antagonist to *B. bassiana* compared to the silt loam soil which contained very low organic matter (< 1 %).

Soil with 7.5 % peat contains more carbon and nitrogen sources than soil without peat which may be responsible for the higher F418 populations observed. Also soil with 7.5 % peat might contain fewer nutrients than soil with 15 % which could limit the growth of antagonist microorganisms and could explain the difference in F418 populations observed between soil with 7.5 and 15 % peat.

C/N ratio of the soils with 0, 7.5 and 15 % peat were different. Smith & Grula (1981) showed that a carbon source is needed for *B*. *bassiana* germination and a nitrogen source is needed for continuous growth otherwise lysis occurs. James (2001) found that a nitrogen source is also necessary for *B*. *bassiana* germination. Based on these studies, it is possible that the C/N ratio of soil with 15 % peat would allow for the germination of F418 but there might not be enough N to balance the C content in order to maintain a continuous growth which could lead to the decline in F418 populations observed.

Soil with 15 % peat was more acidic than the two other soils and this might also be responsible for the decrease in F418 populations. However, Rousk *et al*. (2010) showed that soil pH had no effect on fungus abundance in soil. Therefore this last hypothesis is less likely than the effect of C/N ratio and the presence of antagonists' hypothesis.

3.4.2.4 Impact of presence of host and non-host insect on F418 persistence

The presence of host and non-host insects in pasteurised soil had a significant impact on F418 populations. However, F418 populations in soil with host insects was not higher than in soil without insects, which was unexpected since the host insects died and supported conidia production, which presumably should have increased the F418 populations. The fungus which grew on the cereal bran could be an antagonist of F418, therefore be responsible for the decrease of the F418 populations during the first four weeks that even conidiation from insect cadavers could not fully compensate. Although the fungal contaminants were not formally identified by morphology and DNA sequencing, the colonies ressembled those typical of *Aspergillus* sp. or *Penicillium* sp. Lingg & Donaldson (1981) found that *P. urticae* was a strong antagonist to *B. bassiana* and when a *P. urticae* filtrate was added to some *B. bassiana* inoculated sterile soil, *B. bassiana* could not be isolated from the soil after 12 d. Majchrowicz *et al.* (1990) found that *Aspergillus clavatus* Desm. (Ascomycota: Eurotiales), a common soil saprophyte, inhibited *B. bassiana* growth. Also the

sampling technique used could be partially responsible for the lack of increase in F418 populations in soil with a host insect. *Tenebrio molitor* cadavers covered in conidia were at the surface of the soil and were not mixed with the soil nor sampled during the soil core sampling, which artificially lowered the F418 populations in soil to what it should have been.

The presence of non-host insects lowered F418 populations in soil. Boucias *et al*. (1988) showed that *B. bassiana* conidia can attach passively to non-host cuticle of fourth instar of *A. gemmatalis*. Ment *et al.* (2012) showed that *M. anisopliae* can germinate on non-host cuticle of ticks (Acari: Ixodidae) but cannot penetrate the cuticle and therefore die after a few days. Attachment to non-host cuticle of *C. zealandica* and non-specific germination could be responsible for the F418 populations decrease observed at 17 weeks. This hypothesis could be confirmed by using microscopy to observe any attachment and germination.

Dromph & Borgen (2001) showed that inoculum viability of the soil borne fungus *Tilletia tritici* (Bjerk.) G. Winter (Basidiomycota: Tilletiales) was highly affected after ingestion by four different collembolan species. *Costelytra zealandica* larval gut contains soil particles (Allison, 1969) meaning that the larvae ingest soil, therefore in soil inoculated with F418, *C. zealandica* would ingest F418 conidia which could affect its viability. There is no published report of the effect of *B. bassiana* ingestion by *C. zealandica* or possible germination on *C. zealandica* cuticle. The hypothesis of F418 being digested by *C. zealandica* larvae could be tested by assessing the recovery of F418 in *C. zealandica* larvae faeces following ingestion. Also, *C. zealandica* larvae gut contained non-sterile field soil and their cuticle surface was not sterile, therefore *C. zealandica* larvae may have carried antagonist microorganisms which could contribute to the lower F418 populations in soil with a non-host insect. This hypothesis could be tested by using *C. zealandica* larvae faeces and larval cuticle washes to inoculate soil with F418 and assess the persistence.

The possibility of a non-specific attachment and germination of F418 on the cuticle of *C. zealandica*, ingestion of F418 by *C. zealandica* larvae or F418 antagonists presents in *C. zealandica* larvae are all likely hypotheses to explain the lower F418 population observed in the presence of non-host insects and could be investigated further.

3.4.2.5 Impact of fertilisers on F418 and F418 *gfp* tr1 persistence in pasteurised soil

F418 and F418 *gfp* tr1 populations were influenced by the type of fertiliser added to the soil. In all experiments in pasteurised soil, F418 and F418 *gfp* tr1 populations decreased

significantly when urea was added and were lower than in soil without fertiliser or with superphosphate. Sarathchandra *et al*. (2001) found that *B. brongniartii* represented 0.8 % of the total fungal population in a non-sterile pasture soil without urea. This percentage decreased with increasing nitrogen application and no population of *B*. *brongniartii* was found when nitrogen was applied at 400 kg/ha. In the same study, they showed that population of *Penicillium* sp. increased significantly from 6.9 % to 10.1 % of the total fungal population in soil with no nitrogen added and with 400 kg N/ha respectively. De Vries *et al.* (2006) found that the fungal/bacterial ratio in a grass-clover mix pasture decreased with increasing nitrogen (as manure) application due to a decrease of fungal biomass whereas the bacterial biomass remained stable. Bittman *et al.* (2005) observed a decrease in both bacterial cell and length of fungal hyphae in non-sterile grassland soil when ammonium nitrate was applied at high rate.

Lingg & Donaldson (1981) found that conidia survival was negatively impacted in non-sterile soil amended with urea and suggested that this was due to the increase of other microorganisms. However, when urea was added to sterile soil inoculated with *B. bassiana*, conidia survival was positively affected. There is no report of the impact of nitrogen fertiliser on microorganisms in pasteurised soil, and given that pasteurised soil contains some microorganisms unlike sterile soil, it is therefore difficult to compare the current results to any other published work. Thus, it is unknown if F418 and F418 *gfp* tr1 populations decreased in pasteurised soil amended with urea because of a stimulation of antagonist microorganisms or due to a direct impact of the high nitrogen content in soil (Treseder, 2008; de Vries *et al*., 2006).

F418 and F418 *gfp* tr1 populations in pasteurised soil were higher in soil with superphosphate than without fertiliser. Single superphosphate (2 $CaSO_4 \cdot 2H_2O$ + $Ca(H_2PO_4)_2 \cdot H_2O$) contains phosphorus, calcium, and sulphur which are all elements involved in fungal development. Phosphate is a component of important molecules and nucleotides such as DNA, RNA and ATP, sulphur is a component of proteins and vitamins and growth and reproduction is often enhanced if calcium is added in the media (Garraway & Evans, 1984). Lotan *et al.* (1976) observed a threefold increase in the growth of *Saccharomyces carlsbergensis* with the addition of 500 mg/L of calcium in the media. The increase in F418 and F418 *gfp* tr1 populations in pasteurised soil amended with single superphosphate may be due to the added nutrients available in soil with superphosphate enhancing the fungal growth compared to soil without fertiliser.

The addition of urea had a strong negative impact on F418 and F418 *gfp* tr1 populations whereas the addition of single superphosphate had a slight positive impact on F418 and

F418 *gfp* tr1 populations. Therefore, it was unsurprising that the addition of both urea and single superphosphate gave intermediate population size between those obtained with urea only and single superphosphate only.

3.4.2.6 Impact of fertilisers on F418 *gfp* tr1 persistence in non-sterile soil

Unlike in pasteurised soil, F418 *gfp* tr1 populations in non-sterile soil were all significantly higher with fertilisers than without fertiliser. Both urea and single superphosphate would add nutrients to the soil which could be used by F418 *gfp* tr1 to maintain its viability and could explain the difference in populations compared to without fertiliser. However, this conflicts with published work which showed that fungal biomass declines in non-sterile soil amended with nitrogen (de Vries *et al.*, 2006; Bittman *et al.*, 2005; Lingg & Donaldson, 1981). It should be noted that de Vries *et al.* (2006) and Bittman *et al.* (2005) studied the impact of nitrogen on fungal biomass in grassland soil in the presence of plants. Microorganisms' growth can be limited by the plant roots uptake of soil nutrients (Jingguo & Bakken, 1997). The current microcosm did not contain plants, which may explain the difference to the results obtained by de Vries *et al.* (2006) and Bittman *et al.* (2005). Lingg & Donaldson (1981) used non-sterile soil amended with 0.1 % N (w/w) equivalent to 0.1 mg N/mg soil which is 1000 fold the amount of N used compared to the current study in which 0.12 mg N/1000 mg of soil was used. The difference in N used could explain the different results obtained.

F418 *gfp* tr1 populations increased significantly in non-sterile soil with P and soil without fertiliser at 8 weeks. Similarly, Quintela *et al.* (1992) and Studdert *et al.* (1990) observed some *B. bassiana* populations increase in non-sterile soil even though some studies reported that germination and growth in non-sterile soil is impossible in the absence of a host arthropod (Walstad *et al.*, 1970; Lingg & Donaldson, 1981; Pereira *et al.*, 1993) due to fungistasis.

The impact of fertilisers on F418 *gfp* tr1 populations in non-sterile compared to pasteurised soil was very different. The main difference between non-sterile and pasteurised soil is the microbial community which is much larger and more diverse in non-sterile soil. Ecological niches are occupied in non-sterile soil, thus a large increase of one type of microorganism would be unlikely whereas pasteurised soil has many empty niches and a fast increase of some microorganism would be possible. The hypothesis of the growth of antagonist microorganisms in pasteurised soil amended with urea is more likely than a direct effect of urea on F418 and F418 *gfp* tr1 populations in pasteurised soil since no such negative effect of urea amendment have been observed in non-sterile soil.

3.4.3 Impact of abiotic and biotic factors on F418 and F418 *gfp* tr1 virulence

Virulence of F418 and F418 *gfp* tr1 was correlated to their population sizes in all experiments in pasteurised soil (Appendix F). Virulence of entomopathogenic fungi is usually correlated to the conidial dose given to the host arthropod up to a certain threshold (Jones *et al*., 1996; Wang *et al*., 2004). However, linear regression analyses were not carried out for each sampling time but with all sampling times together and the global analysis might not have detected any lack of correlation for a specific sampling time. F418 populations in pasteurised soil amended with P were significantly higher than in soil amended with N at 12 weeks; however the virulence of F418 populations from these two treatments were not significantly different at 12 weeks (Appendix F). Therefore, F418 and F418 *gfp* tr1 populations' virulence might not have been correlated to F418 and F418 *gfp* tr1 populations' levels for each sampling time.

Shah *et al*., (2005) showed that *M. anisopliae* virulence depended on the media on which the fungus was grown. The lack of significant difference in virulence of F418 in pasteurised soil amended with P and N at 12 weeks could have been due to a difference of virulence of individual conidium or a difference in propagule type present (conidia *vs*. mycelia) but the soil dilution plating method used does not enable discrimination of these different propagules. Assessing the virulence of F418 and F418 *gfp* tr1 in soil suspensions at same CFU concentration would have been useful to determine if the propagule present had a different virulence; however this was not technically feasible. The virulence experiments showed that in general, F418 and F418 *gfp* tr1 LT_{50} was negatively correlated to F418 and F418 *gfp* tr1 populations in soil.

3.4.4 F418 *gfp* transformant as a tool for *in vivo* experiment

F418 and F418 *gfp* tr1 populations were studied in pasteurised soil with different fertilisers and the populations of the two strains were compared for each time and treatment and showed no difference between 0 and 8 weeks. The only difference appeared at 13 weeks with F418 *gfp* tr1 populations in soil without fertiliser and with P being lower than F418 populations in the same conditions. This experiment showed that the F418 *gfp* tr1 populations behaviour in soil in similar to F418 which confirmed the conclusion drawn in Chapter 2 about the suitability of F418 *gfp* transformant as a substitute to F418.

F418 *gfp* tr1 was used in this chapter as it was shown in Chapter 2 to be more similar to F418 than F418 *gfp* tr3 was. However, attempts to observe the fungus in soil suspensions using fluorescence microscopy (not detailed in this chapter) failed due to the lack of brightness of F418 *gfp* tr1 compared to F418 *gfp* tr3. F418 *gfp* tr3 was used in subsequent soil experiments.

3.4.5 Conclusion

The following conclusions can be drawn from the results presented in this chapter:

- All the factors tested in pasteurised soil had an impact on F418 and F418 *gfp* tr1 populations.
- A common hypothesis emerged in all factors tested being that it was not the direct effect of the factor but the indirect effect on antagonist microbes (or lack of) which impacted F418 populations.
- The impact of soil moisture on F418 persistence in soil was chosen to be studied more thoroughly as all literature agrees on the general effect of soil moisture level on *B. bassiana* populations in soil and hypothesised that the indirect effect of other soil microorganisms is responsible for the observations made; however no studies confirmed the hypothesis of the impact of other soil microorganisms and this needs to be investigated by studying the soil microbial communities present in the soils at different moisture levels.
- The impact of the presence of host, non-host and no insect was chosen to be studied more thoroughly as the results obtained in this chapter were of interest with F418 populations in pasteurised soil with non-host insect being lower than without insect and there is no literature about *B. bassiana*/non-host arthropod interactions in soil.
- When the effect of fertilisers on F418 *gfp* tr1 populations was tested in both pasteurised and non-sterile soil, the results were divergent; which demonstrated that pasteurised soil cannot be used to draw conclusion about the fate of F418 in natural pasture soil. However, the use of pasteurised soil improved understanding of the potential mechanisms involved such as the presence of different microorganisms.
- F418 *gfp* tr1 populations were similar to F418 populations in the same soil conditions, therefore F418 *gfp* transformant can be used a substitute to F418 *in vivo*.

- The low level of fluorescence emitted by F418 *gfp* tr1 means that the brighter F418 *gfp* tr3 should be used in subsequent experiments to enable observations under fluorescent microscopy.

Chapter 4
Effects of soil moisture on persistence and propagule dynamics of *Beauveria bassiana* F418 *gfp* tr3

4.1 Introduction

In Chapter 3, *B. bassiana* F418 populations were negatively affected by increasing moisture level in soil. Similar observations were made by Lingg & Donaldson (1981), Studdert *et al*. (1990) and Quintela *et al.* (1992) and all suggested that the presence of antagonistic bacteria or fungi could be having a detrimental effect on *B. bassiana*. However, none studied the microbial communities in soil at different moisture levels to confirm the hypothesis that *B. bassiana* populations were lower at high moisture level because of an increase of microbial antagonists in wetter soil.

Also in Chapter 3, a significant increase in *B. bassiana* F418 populations in pasteurised soil was observed during the first two weeks at all moisture levels. Lingg & Donaldson (1981) and Quintela *et al.* (1992) observed similar *B. bassiana* CFU increase and they hypothesised that *B. bassiana* could grow in soil but they did not study the different propagule types in soil and therefore did not know if the CFU increase observed was due to mycelial growth or conidia formation. Pereira *et al.* (1993) observed mycelial growth and conidiation in sterile soil inoculated with *B. bassiana* conidial suspension whereas no growth could be observed in the same condition in non-sterile soil. There is no publication on the effect of soil moisture on *B. bassiana* propagules types and their dynamics in soil, therefore, it was unknown if the *B. bassiana* F418 populations increase observed in pasteurised soil in Chapter 3 was due to mycelial growth or conidiation.

Different gravimetric water contents were used in pasteurised soil in Chapter 3, however the soil pasteurisation might have changed the water retention capacity compared to non-sterile soil and the use of water contents does not reflect the water availability in different soil types. Therefore, moisture release curves were drawn for pasteurised and non-sterile Templeton silt loam soil and both water contents and water potentials were used in this Chapter's experiments.

The first objective covered in this chapter was to determine the effects of high and low soil moisture on *B. bassiana* F418 *gfp* tr3 populations in pasteurised soil, to confirm the results obtained in Chapter 3, and in non-sterile soil to investigate if the effects observed in pasteurised soil were similar when applied to a more natural environment. A second objective for this chapter was to investigate the fungal and bacterial communities present in soil with different moisture levels using single strand conformational polymorphism (SSCP) to examine the hypothesis that the lower persistence of *B. bassiana* in wetter soil is correlated to changes in microorganisms present in the soil. A final objective was to investigate the dynamics of the different propagule types of *B. bassiana* F418 *gfp* tr3 in soil (mycelia and conidia) using a modified soil sandwich method in pasteurised and non-sterile soil under different moisture levels.

4.2 Methods

4.2.1 Determination of moisture release curves for pasteurised and non-sterile Templeton silt loam soil

Soil moisture release curves were drawn for pasteurised and non-sterile Templeton silt loam soil to investigate if the pasteurisation changed the water retention capacity of the soil and therefore determine if water contents or water potentials should be used in soil experiments with pasteurised and non-sterile Templeton silt loam soil.

Pasteurised Templeton silt loam soil was collected and treated as described in section 3.2.1 and non-sterile soil was collected and treated as described in section 3.2.1 except for the sampling date (July 2009).

Soil moisture release curves of pasteurised and non-sterile Templeton silt loam soil were determined using Equi-pf (Streat instruments, Christchurch, New Zealand) for measurements between 0 and -0.1 bar and pressure plate extractors (Soil moisture equipment corporation, Santa Barbara, CA, USA) for measurement between -0.5 and -10 bar. Two pressure plate extractors were used, the first one for pressures ranging from -0.5 to -3 bar and the second for pressures ranging from -5 to -10 bar.

Metal rings (98 mm Ø × 20 mm high) filled with saturated pasteurised or non-sterile Templeton silt loam soil were placed on the Equi-pf porous plate and the moisture release curve measured between 0 and -0.1 bar. Once the soil moisture release curve was measured, soil from the ring was recovered and the weight of wet and dry soil (after 24 h at 105 °C) recorded. There were three individual replicate rings for each soil.

With the pressure plate extractors, plastic rings (35 mm Ø × 10 mm high) were filled with saturated pasteurised and non-sterile Templeton silt loam soil and held in the ring using a nylon mesh secured with a rubber band. There were three replicate rings for each soil and all the rings were placed in the pressure plate extractor. A tension of -0.5 bar was applied first. Once the soil reached the applied tension, the rings were removed and a weight measurement made. The rings were replaced and the process repeated for tensions of -1 and -3 bar. The rings were moved to the second pressure plate extractor for pressure applied at -5, -7 and -10 bar. After -10 bar was applied, full ring weight was recorded as well as wet soil from the ring and dry soil (after 24 h at 105 °C) allowing the soil moisture release curves to be plotted.

Data from the Equi-pf and the pressure plate extractors were plotted to draw soil moisture release curves for pasteurised and non-sterile Templeton silt loam soil between -0.1 and -10 bar.

4.2.2 Evaluation of F418 *gfp* tr3 persistence in soil at different moisture level

4.2.2.1 Effects of soil water content

The objective of this experiment was to determine the effect of low and high moisture levels, using different gravimetric water contents, on F418 *gfp* tr3 persistence in pasteurised and non-sterile soil, and assess if the effects were similar to the results obtained in Chapter 3 and if the results were similar in pasteurised and non-sterile soil.

Experiments were carried out in 60 mL screw cap containers (LabServ, New Zealand) filled with 15 g of dry pasteurised soil (treatment described in section 3.2.1) or equivalent using non-sterile soil, inoculated with 2 x 10^5 F418 *gfp* tr3 conidia suspension/g DWS. F418 *gfp* tr3 conidia were produced and their viability checked using the protocol described in section 2.2.1.2. The effect of water content on the persistence of F418 *gfp* tr3 in pasteurised and non-sterile soil was determined by wetting the soils to either 22 % GWC or 31 % GWC

(Table 4.2). The soil was thoroughly mixed with the water and conidial suspension and incubated at 15 °C in the dark using a RCBD. There were four replicate containers per treatment. Three additional replicate containers were prepared for each treatment without addition of F418 *gfp* tr3 to be sampled at the end of the experiment for studies on the soil bacterial and fungal communities (section 4.2.2.5). The pasteurised soil used was as described in section 3.2.1 and the non-sterile soil was collected in July 2009 and treated as described in the section 3.2.1. The experiment was set up at the beginning of August 2009.

4.2.2.2 Assessment of F418 *gfp* tr3 persistence in soil

Containers were destructively sampled at 0, 1, 2, 3, 4, 6, 8 and 10 weeks. Persistence was monitored using a soil dilution plating method derived from Davet & Rouxel (2000). Total soil from each container was mixed with 135 mL of 0.01 % SDWA in a 250 mL screw cap container (LabServ, New Zealand); this constituted the first soil dilution (10^{-1} dilution). The protocol was then as described in section 3.2.4. Serial dilutions from 10^{-2} to 10^{-4} were prepared and 100 µL of each dilution spread onto a Petri plate of 10 % SDA+A+Hyg (Appendix A). There were duplicate plates for each sample and dilution. The plates were arranged in a RCBD and incubated at 20 °C in the dark for two weeks before counting the CFU. Persistence was expressed as the number of CFU recovered per g DWS.

4.2.2.3 Effects of soil water potential

The objective of this experiment was to determine the effect of low and high moisture levels, using different water potentials, on F418 *gfp* tr3 persistence in pasteurised and non-sterile soil, and assess if the effects were similar to the results obtained in Chapter 3 and if the results were similar in pasteurised and non-sterile soil.

An experiment was set up and sampled using the same protocol as described in the previous sections except for the following details: the effect of water potential on the persistence of F418 *gfp* tr3 in pasteurised and non-sterile soil was determined by hydrating the soils to either -0.33 bar or -5 bar with SDW (Table 4.2). Containers were destructively sampled at 0, 1, 2, 4, 6, 10, 14 and 18 weeks. The non-sterile soil was collected in March 2010 and the experiment was set up the same month.

4.2.2.4 Observation of soil suspensions using fluorescence microscopy

The objective of this experiment was to observe F418 *gfp* tr3 in soil suspensions to determine which propagule types were present in the soils as a function of the soil type, the moisture level, and the sampling time.

Soil suspensions (10^{-1} dilution) were observed by fluorescence microscopy to determine the propagule type present in the soil. Soil dilution from fungal assessments in 250 mL containers were shaken by hand and left to rest for two minutes to allow soil particles of large size to sink; 20 µL was placed onto a microscope slide and covered by a coverslip. Slides were observed using fluorescence microscopy (Olympus BX50, magnification x400, excitation filter Olympus U-MWIB3, software Olympus Olysia and DP2-BSW, camera Olympus DP12 and DP72, Olympus Corp., Tokyo, Japan). Soil suspensions from at least one replicate from each treatment were observed at every sampling between 0 and 6 weeks during the experiment with different water contents (2009) only. Photographs of propagules were taken when found on the slide. The tone curves of the colours were adjusted in Corel Photo Paint 12 (Corel Corporation, Canada) to enhance the contrast of the F418 *gfp* tr3 propagules.

4.2.3 Comparison of fungal and bacterial communities in soil with different water content and potential using SSCP

The objective of this experiment was to determine the fungal and bacterial soil communities' patterns function of the soil type, the moisture levels and time to establish if a correlation existed between the soil communities and the soil type/moisture level.

Single-strand conformational polymorphism (SSCP) was used to detect differences in bacterial and fungal communities in soil with different water contents or water potentials. Soil containers prepared for the study of soil fungal and bacterial communities (4.2.2.1 and 4.2.2.3) were sampled at the start and end of each experiment and the 0.5 g soil samples were stored at - 80 °C in 1.2 mL sterile screw cap tubes until DNA extraction.

DNA was extracted from a 0.25 g of soil subsample using PowerSoil® DNA isolation kit (MO BIO Laboratories Inc., CA, USA) as per manufacturer's instructions. DNA quality was confirmed by separating 5 µL of the extracted DNA mixed with 2 µL of 6 X loading dye

(0.025 % bromophenol blue, 0.025 % xylene cyanol, 40 % w/v sucrose) on a 1 % agarose gel (Bioline, Meridian Life Science Incorporation, OH, USA) by electrophoresis at 5 V/cm for 45 min alongside a 1 kb+ DNA ladder (Invitrogen, Life Technologies, CA, USA). Gels were stained in 0.0005 % ethidium bromide in 1 X TAE (40 mM Tris acetate, 1 mM EDTA and 20 mM acetic acid) for 15 min, rinsed in dH_2O and photographed under UV light using VersaDoc™ Imaging System (Model 3000, Bio-Rad, CA, USA). DNA was stored at - 20 °C.

DNA amplifications of the bacterial 16S region were performed using U806Ir and B342If (Table 4.1) and of the fungal ITS region using primers ITS3 and ITS4 (Table 4.1).

Table 4.1 List of primers used for the amplification of fungal and bacterial genes

Primer	Sequence (5'-3')	Reference
B342If	CTACGGGIGGCIGCAGT	Watanabe *et al*., 2001
U806Ir	GGACTACCIGGGTITCTAA	Takai & Horikoshi, 2000
ITS3	GCATCGATGAAGAACGCAGC	White *et al*., 1990
ITS4	TCCTCCGCTTATTGATATGC	White *et al*., 1990

PCR were performed in a total volume of 25 µL containing 1 × PCR reaction buffer with 1.5 mM $MgCl_2$ (Roche, Basel, Switzerland), 200 µM of each dNTP (Roche, Basel, Switzerland), 5 pM of each primer (Invitrogen, Life Technologies, CA, USA), 5 µg of bovine serum albumin (10 mg/mL, New England BioLabs Inc., USA) and 0.625 U Taq DNA polymerase (Roche, Basel, Switzerland). In each tube, 5 ng of isolated DNA was added and a DNA-free control was included in every PCR run to ensure absence of contamination. An Applied Biosystems Veriti thermocycler (Veriti 96 well thermal cycler, Applied Biosystems, Life Technologies, CA, USA) was used for the amplifications. The temperature profile used for the B342If/U806Ir primers was 2 min at 94 ºC followed by 32 cycles of 1 min at 94 ºC, 1 min at 55 ºC and 1 min at 72 ºC. The 32 cycles were followed by 5 min at 72 ºC (Hori *et al*., 2006). The temperature profile used for the ITS3/ITS4 primers was 3 min at 95 ºC followed by 35 cycles of 45 s at 94 ºC, 45 s at 56 ºC and 45 s at 72ºC. The 35 cycles were followed by 7 min at 72 ºC. Amplified products were visualised on agarose gels as described in the previous paragraph.

Before SSCP, 2 µL of bacterial or fungal PCR products were mixed with 30 µL of denaturing loading dye (Appendix I) and incubated at 99 °C for 7 min in an Applied Biosystems Veriti thermocycler (Veriti 96 well thermal cycler, Applied Biosystems, Life Technologies) and immediately placed on ice. The samples (20 µL) and a 1 kb plus DNA ladder (Invitrogen, Life

Technologies, CA, USA) were separated by electrophoresis in a MDE gel matrix (Appendix I). Gels were run at 200 V for 17 h at 20 °C (Grant LVF6, Grant Instruments Ltd, UK) in a Protean® II xi cell apparatus (Bio-Rad Laboratories Inc.) with 1 X TBE buffer (Appendix I). Gels were silver stained using a method derived from Sanguinetti *et al.* (1994) by immersion in fixer (10 % v/v ethanol, 0.5 % v/v acetic acid) (Appendix I) for 3 min followed by silver staining for 5 min in 10 % v/v ethanol, 0.5 % v/v acetic acid and 0.2 % w/v silver nitrate (Appendix I). Gels were then rinsed in dH_2O and washed in dH_2O for 2 min before developing in 3 % NaOH and 0.1 % CH_2O (Appendix I) for approximately 25 min. Gels were then placed in fixer for 5 min and rinsed in dH_2O (Bassam *et al*., 1991; Benbouza *et al*., 2006). After staining, gels were scanned between two sheets of cellulose acetate on a Canon photocopier/scanner.

Bands of interest were excised from the gel using a scalpel blade sterilised using 70 % ethanol between each cut and each band was cut in two pieces and each piece stored in a 0.2 mL sterile tube (Axygen, CA, USA) at -20 °C. DNA was extracted from the MDE gel slice using the Schwieger & Tebbe (1998) method. Fungal and bacterial DNA was reamplified as described previously except that the annealing temperature was increased to 61 °C for both primers sets instead of 55 °C and 56 °C for the bacterial primers and fungal primers, respectively. Reamplified products were visualised on agarose gels as described previously and selected products were sequenced using U806Ir for the bacterial product and ITS4 for the fungal products at the Lincoln University Sequencing Facility (Lincoln, New Zealand). Sequences obtained were manually checked and trimmed of any ambiguous sequence. The nucleotide Basic Local Alignment Search Tool (BLAST®, NCBI) (Altschul *et al*., 1990) was used to obtain the closest bacterial or fungal match for each sequence based on the maximum identity and E values returned.

4.2.4 Evaluation of F418 *gfp* tr3 population dynamics in soil with different water content and water potential

4.2.4.1 Membrane sandwich in soil with different water content

The objective of this experiment was to monitor F418 *gfp* tr3 propagule dynamics in a membrane sandwich in pasteurised and non-sterile soil at different moisture levels (using water contents) and determine quantitatively the dynamics of washed propagules inside the

membrane sandwiches and qualitatively the dynamics of mycelium formation inside the membrane sandwiches.

The experiment was done in 90 × 25 mm plastic Petri dishes (LabServ, New Zealand) filled with a first layer of 30 g of dry pasteurised soil or equivalent using non-sterile soil moistened to the desired level. The soil sandwich method was modified from Grose *et al.* (1984). A membrane sandwich was created using two nylon transfer membranes of approximately 35 × 40 mm with 0.2 µm pore size (Biodyne A, Pall, USA) held in a photographic slide frame (Fig 4.1). Ten µL of a 1 x 10^8 F418 *gfp* tr3 conidial suspension was placed in the middle of the membrane sandwich (1 x 10^6 conidia per sandwich). F418 *gfp* tr3 conidia were produced and their viability checked using the protocol described in 2.2.1.2. After inoculation, the membrane sandwich was placed on top of the first layer of soil and covered with a second layer of 30 g of dry pasteurised soil or equivalent using non-sterile soil moistened to the desired level (Fig. 4.1). Petri dishes were closed and moisture was maintained by wrapping the dish joint using Parafilm™ (Pechiney Plastic Packaging Company, USA). The effect of water content on the population dynamics of F418 *gfp* tr3 in pasteurised and non-sterile soil was determined by wetting the soils at either 22 % GWC or 31 % GWC (Table 4.2).

There were four replicate dishes for each treatment and sampling time. Petri dishes were incubated at 15 °C using a split plot design. The pasteurised soil used was as described in section 3.2.1 and the non-sterile soil was collected in July 2009 and treated as described in the section 3.2.1. The experiment was set up in October 2009.

Figure 4.1 Nylon membranes sandwich held inside a photographic slide frame between two layers of soil in a Petri dish.

4.2.4.2 Membrane sandwich in soil with different water potential

The objective of this experiment was to monitor F418 *gfp* tr3 propagule dynamics in a membrane sandwich in pasteurised and non-sterile soil at different moisture levels (using water potentials) and determine quantitatively the dynamics of washed propagules inside the membrane sandwiches and qualitatively the dynamics of mycelium formation inside the membrane sandwiches.

An experiment was set up using the same protocol than as in section 4.2.4 apart from the following details. The nylon transfer membrane used was 0.45 µm pore size instead of 0.2 µm (Biodyne A, Pall, USA) to overcome the more sterile environment created by the 0.2 µm membrane. The effect of water potential on the population dynamics of F418 *gfp* tr3 in pasteurised and non-sterile soil was determined by wetting the soils at either -0.33 bar or -5 bar with SDW (Table 4.2). The non-sterile soil was collected in March 2010 and the experiment was set up in April 2010.

4.2.4.3 Assessment of washed propagules from membrane sandwiches

Dishes were sampled at 0, 1, 2, 3, 4, 7, 10 and 21 weeks. The initial inoculum level was not measured for the experiment in membrane sandwich in soil at different water content (4.2.4.1) and was considered as the applied amount of 1×10^6 conidia per sandwich.

The membrane sandwich was recovered from the soil, opened and the membranes placed in a 10 mL centrifuge tube containing 5 mL of sterile 0.01 % v/v Triton® X-100 (BDH, Poole, UK). Tubes were vortexed for approximately one min and serial dilutions (10^{-1} to 10^{-4}) were prepared. One hundred µL of each dilution was plated onto 10 % SDA+A+Hyg. There were duplicate plates for each membrane sandwich and dilution. The plates were arranged in a RCBD and incubated at 20 °C in the dark for two weeks before counting the CFU.

4.2.4.4 Assessment of mycelial growth on the membranes

Following the washing of the membranes as described in the previous paragraph, membranes were removed from the tube and left to air dry. Once dried, membranes were stained in a bath of lactophenol blue solution (Merck, USA) for two min and rinsed in water for five min using a method derived from Wakelin *et al.* (1999). When a F418 *gfp* tr3 mycelial

growth ring was present, its diameter was measured at two perpendicular axes (Fig. 4.2). The precision of the diameter measurement was made to within 0.5 mm.

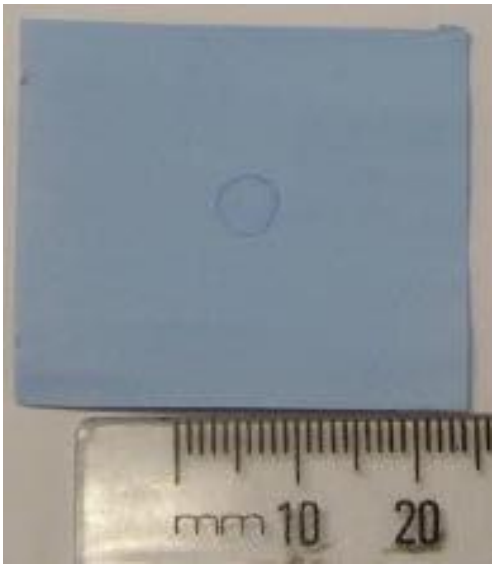

Figure 4.2 Nylon membrane from pasteurised soil at -5 bar after staining with lactophenol solution and showing a mycelial growth ring.

4.2.5 Statistical analysis

Data for soil moisture realease curves were analysed using a general ANOVA with treatment factors being soil types and pressures (with a linear polynomial contrast). All CFU recovery data were $\log_{10}$ transformed to achieve a normal distribution essential to the use of ANOVA (Olsen, 2003). A split plot ANOVA was carried out, with treatment factors being the treatments (soil moisture and soil type) and time, with a linear polynomial contrast specified where appropriate. Significance was evaluated at $P < 0.05$ for all analyses and mean separation was executed by the unprotected LSD test. All data analyses were performed using the statistical software Genstat version 12.2 (VSN International). All statistical results tables are presented in Appendix K.

4.3 Results

4.3.1 Pasteurised and non-sterile Templeton silt loam soil moisture release curves

Soil moisture release curves for pasteurised and non-sterile Templeton silt loam soil obtained using Equi-pf (Streat instruments, Christchurch, New Zealand) and pressure plate extractors (Soil moisture equipment corporation, Santa Barbara, CA, USA) between -0.1 and -10 bar are presented in Fig. 4.3. Pasteurised and non-sterile Templeton silt loam moisture release curves were significantly different at all pressure point except -0.1 bar. Water content in pasteurised soil was significantly lower than in non-sterile soil at -0.5, -1, -3, -5, -7 and -10 bar.

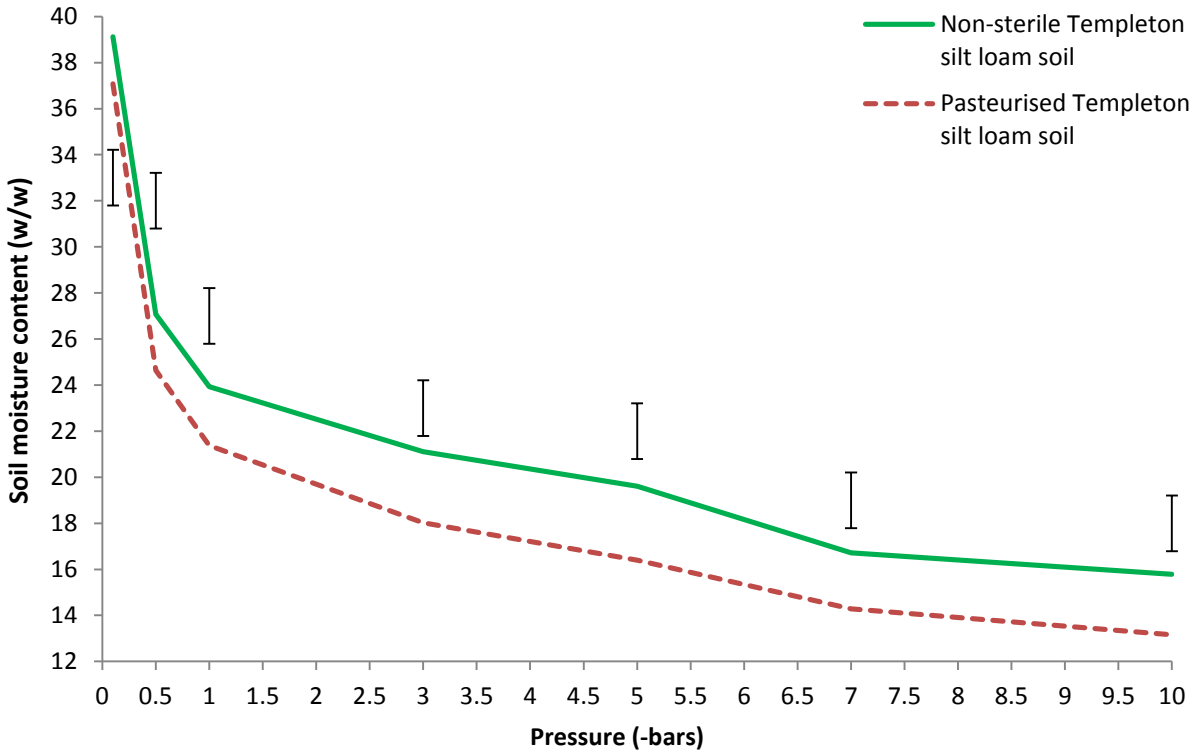

Figure 4.3 Soil moisture release curves for pasteurised and non-sterile Templeton silt loam soil between -0.1 and -10 bar. Vertical bars are LSD ($P < 0.05$).

The equivalence between water potential to gravimetric water content and field capacity in pasteurised and non-sterile Templeton soil used in the different experiments is in Table 4.2.

Table 4.2 Equivalence between soil gravimetric water content, water potential and percentage field capacity in pasteurised and non-sterile Templeton silt loam soil

Water potential (bar)	% gravimetric water content		% field capacity	
	Pasteurised soil	Non-sterile soil	Pasteurised soil	Non-sterile soil
-0.33	**31 %**	34.5 %	**100 % FC**	100 % FC
-0.42		**31 %**		**90 % FC**
-0.50	24.6 %	27.1 %	79 % FC	79 % FC
-0.85	**22 %**		**71 % FC**	
-2	19.7 %	22.5 %	64 % FC	65 % FC
-2.15		**22 %**		**64 % FC**
-5	16.4 %	19.6 %	53 % FC	57 % FC
-8	14 %		45 % FC	

4.3.2 Effect of soil moisture level on persistence of F418 *gfp* tr3 in soil

4.3.2.1 Persistence at different water content

The effect of soil water content on F418 *gfp* tr3 populations was assessed over 10 weeks (Table 4.3 and Fig 4.4). F418 *gfp* tr3 populations in pasteurised soil at 22 % GWC increased significantly during the first four weeks compared to the INIL and were maintained significantly high during the whole experiment, with 392 % of the INIL recovered at 10 weeks. F418 *gfp* tr3 populations in pasteurised soil at 31 % GWC were not significantly different from the INIL at all sampling times except at week 1 where the populations were significantly lower. F418 *gfp* tr3 populations in pasteurised soil at 22 % GWC was significantly higher than in pasteurised soil at 31 % GWC between 1 and 10 weeks.

In non-sterile soil, there was no significant difference between F418 *gfp* tr3 populations recovered from soil at 22 % and 31 % GWC during the whole experiment. F418 *gfp* tr3 populations in non-sterile soil at both moisture levels decreased during the whole experiment and were significantly lower than the INIL at 8 and 10 weeks. F418 *gfp* tr3 populations in non-sterile soil were 61 % and 58 % of the INIL at 10 weeks in soil at 31 % and 22 % GWC, respectively. F418 *gfp* tr3 recovery in pasteurised soil at 22 % GWC was significantly higher than in non-sterile soil at 22 % GWC at all sampling times (except week 0). F418 *gfp* tr3 recovery in pasteurised soil at 31 % GWC was significantly higher than in non-sterile soil at 31 % GWC at 4, 8 and 10 weeks.

Table 4.3 F418 *gfp* tr3 populations recovered (CFU/g DWS and $\log_{10}$ value) in pasteurised and non-sterile soil at 22 and 31 % GWC held at 15 °C over 10 weeks.

CFU/g DWS **($\log_{10}$)**	Week 0	Week 1	Week 2	Week 3	Week 4	Week 6	Week 8	Week 10
Pasteurised soil at 31 % GWC	3.27×10^5 **(5.51)**	2.06×10^5 **(5.31)**	3.32×10^5 **(5.52)**	3.27×10^5 **(5.51)**	3.64×10^5 **(5.56)**	2.89×10^5 **(5.46)**	3.48×10^5 **(5.54)**	2.78×10^5 **(5.44)**
Pasteurised soil at 22 % GWC	3.60×10^5 **(5.56)**	8.36×10^5 **(5.92)**	1.03×10^6 **(6.01)**	7.82×10^5 **(5.89)**	1.21×10^6 **(6.08)**	1.21×10^6 **(6.08)**	1.29×10^6 **(6.11)**	1.41×10^6 **(6.15)**
Non-sterile soil at 31 % GWC	2.94×10^5 **(5.47)**	2.67×10^5 **(5.43)**	2.65×10^5 **(5.42)**	2.57×10^5 **(5.41)**	2.38×10^5 **(5.38)**	2.03×10^5 **(5.31)**	1.89×10^5 **(5.28)**	1.78×10^5 **(5.25)**
Non-sterile soil at 22 % GWC	2.91×10^5 **(5.46)**	2.06×10^5 **(5.31)**	2.11×10^5 **(5.32)**	2.36×10^5 **(5.37)**	2.49×10^5 **(5.40)**	1.96×10^5 **(5.29)**	1.63×10^5 **(5.21)**	1.68×10^5 **(5.23)**
LSD ($P < 0.05$)				**0.18**				

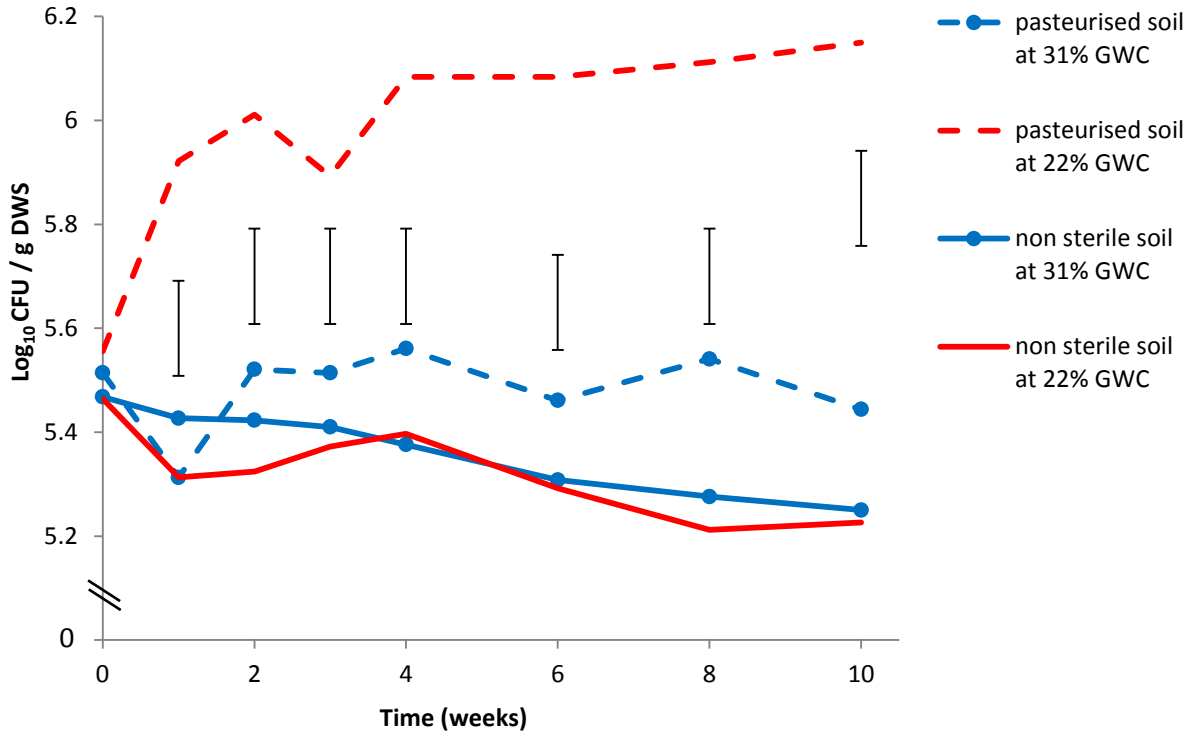

Figure 4.4 F418 *gfp* tr3 populations recovered ($\log_{10}$ CFU/g DWS) in pasteurised and non-sterile soil at 22 and 31 % GWC held at 15 °C over 10 weeks in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

4.3.2.2 Persistence at different soil water potential

The effect of water potential on F418 *gfp* tr3 in pasteurised soil and non-sterile soil was monitored for 18 weeks (Table 4.4 and Fig. 4.5). F418 *gfp* tr3 populations in pasteurised soil at -5 bar increased significantly during the first 2 weeks compared to the INIL and were maintained significantly high during the whole experiment, with 352 % of the INIL recovered at 18 weeks. F418 *gfp* tr3 populations in pasteurised soil at -0.33 bar decreased continually during the whole experiment and were significantly lower than the INIL at all sampling times; it was 29 % of the INIL at 18 weeks. F418 *gfp* tr3 populations in pasteurised soil at -5 bar were significantly higher than at -0.33 bar between 1 and 18 weeks.

In non-sterile soil, there was no significant difference between F418 *gfp* tr3 populations recovered from soil at -0.33 and -5 bar during the whole experiment. F418 *gfp* tr3 populations in non-sterile soil at both moisture levels decreased during the whole experiment and were significantly lower than the INIL between 4 and 18 weeks in soil at -0.33 bar and at 4, 10, 14 and 18 weeks in soil at -5 bar. F418 *gfp* tr3 populations in non-sterile soil at 18 weeks were 52 % the INIL at -0.33 bar and 59 % the INIL at -5 bar.

F418 *gfp* tr3 population in pasteurised soil at -5 bar was significantly higher than in non-sterile soil at -5 bar at all sampling times. F418 *gfp* tr3 population in pasteurised soil at -0.33 bar was significantly lower than in non-sterile soil at -0.33 bar at 1, 2 and 6 weeks.

Table 4.4 F418 *gfp* tr3 populations recovered (CFU/g DWS and $\log_{10}$ value) in pasteurised and non-sterile soil at -0.33 and -5 bar held at 15 °C over 18 weeks.

CFU/g DWS **($\log_{10}$)**	Week 0	Week 1	Week 2	Week 4	Week 6	Week 10	Week 14	Week 18
Pasteurised soil at -0.33 bar	1.40×10^5 **(5.15)**	4.79×10^4 **(4.68)**	6.68×10^4 **(4.83)**	7.08×10^4 **(4.85)**	5.58×10^4 **(4.75)**	7.78×10^4 **(4.89)**	4.49×10^4 **(4.65)**	4.05×10^4 **(4.61)**
Pasteurised soil at -5 bar	1.46×10^5 **(5.17)**	4.50×10^5 **(5.65)**	6.18×10^5 **(5.79)**	6.17×10^5 **(5.79)**	6.19×10^5 **(5.79)**	9.38×10^5 **(5.97)**	6.78×10^5 **(5.83)**	5.15×10^5 **(5.71)**
Non-sterile soil at -0.33 bar	1.43×10^5 **(5.16)**	1.40×10^5 **(5.15)**	1.13×10^5 **(5.05)**	9.23×10^4 **(4.97)**	8.87×10^4 **(4.95)**	7.87×10^4 **(4.90)**	6.71×10^4 **(4.83)**	7.38×10^4 **(4.87)**
Non-sterile soil at -5 bar	1.30×10^5 **(5.11)**	1.38×10^5 **(5.14)**	1.26×10^5 **(5.10)**	8.09×10^4 **(4.91)**	1.06×10^5 **(5.03)**	7.83×10^4 **(4.89)**	8.02×10^4 **(4.90)**	7.60×10^4 **(4.88)**
LSD ($P < 0.05$)				**0.19**				

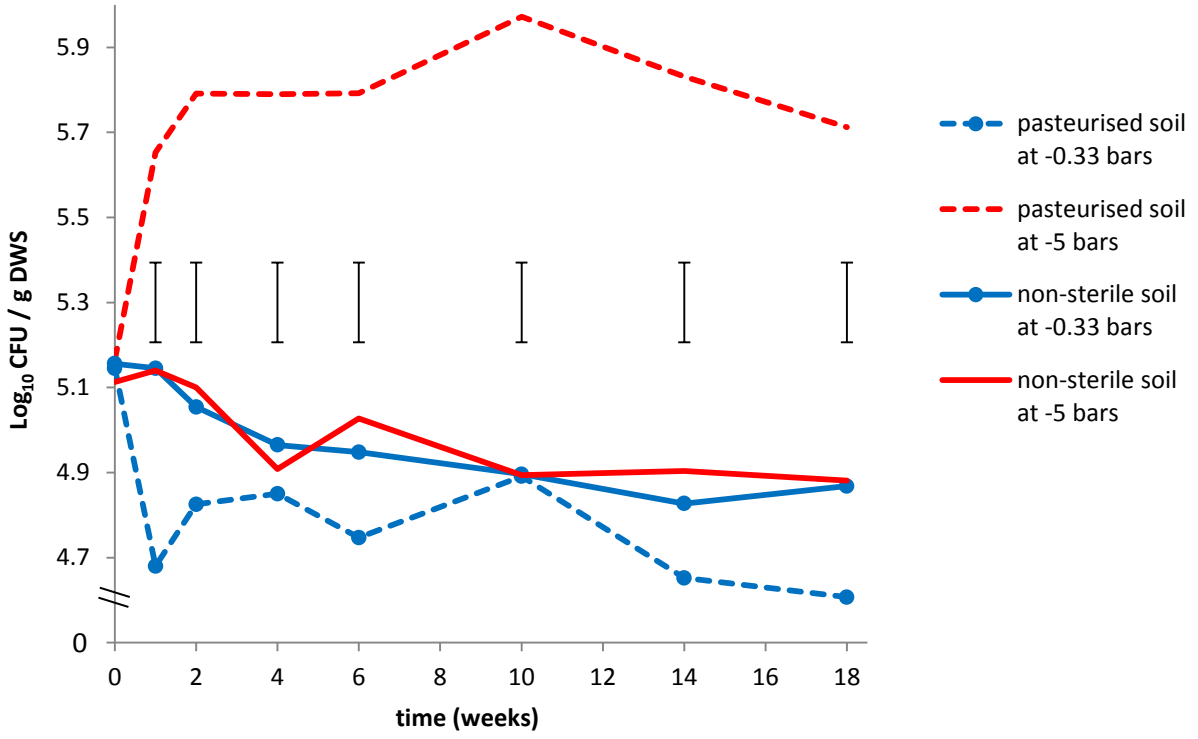

Figure 4.5 F418 *gfp* tr3 populations recovered ($\log_{10}$ CFU/g DWS) in pasteurised and non-sterile soil at -0.33 and -5 bar held at 15 °C over 18 weeks in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

4.3.2.3 Soil suspension observation under fluorescence microscopy

Individual conidia could be observed in soil suspensions (Fig. 4.6a) from all treatments, however the observation was difficult due to the faintness of the fluorescence emitted by the F418 *gfp* tr3 conidia and very few conidia could be observed compared to the number present in the soil dilutions (i.e. around 400 conidia were theoretically present in the 20 µL soil suspension on the slide at week 0 but only around 10 could be observed).

A probable F418 *gfp* tr3 germinated conidium was observed from a suspension of pasteurised soil at 22 % GWC at week 2 (Fig 4.6b). A probable F418 *gfp* tr3 piece of mycelium was observed from a suspension of pasteurised soil at 22 % GWC at week 3 (Fig 4.6d) and clusters of F418 *gfp* tr3 conidia could be observed in pasteurised soil at 22 % GWC (Fig. 4.6c) and 31 % GWC at 4 and 6 weeks.

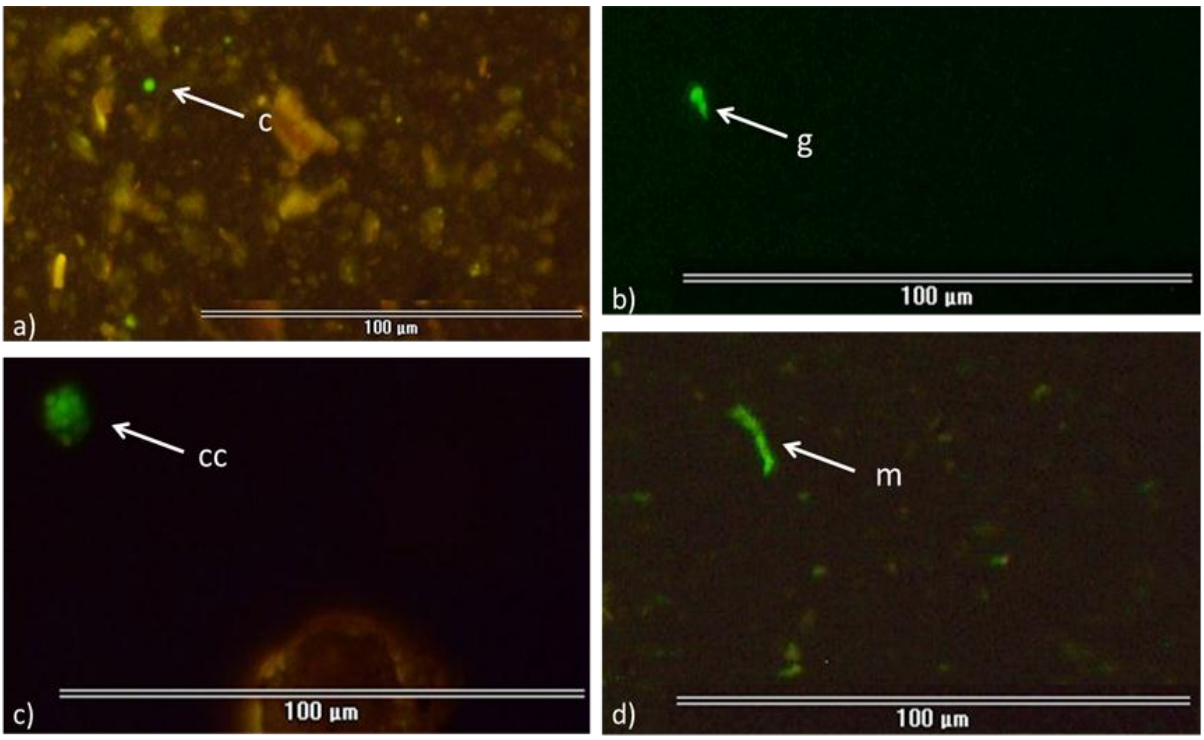

Figure 4.6 Fluorescent microscopy photographs of F418 *gfp* tr3 (a) conidium; (b) a germinated conidium; (c) a cluster of conidia and (d) a piece of mycelium. (Enhanced contrast used to adjust photos)

4.3.3 Fungal and bacterial community changes in soil

4.3.3.1 Bacterial and fungal community changes in soil at different water contents

The SSCP banding patterns of bacteria recovered from pasteurised soil at different water contents changed from the beginning to the end of the experiment and the patterns were also different at the end of the experiment between soil at 22 and 31 % GWC (Fig 4.7 and Table 4.5). Numerous bacterial bands present in soil at 31 % GWC such as bands 102, 94 and 91 were absent in soil at 22 % GWC. Some bacterial bands were common to both water contents in pasteurised soil at the end of the experiment such as bands 106 and 104.

There was no difference in bacterial band patterns in non-sterile soil with different soil water contents between different treatments and time (Fig. 4.7). As expected, the number of bacterial bands was much greater in non-sterile soil than in pasteurised soil.

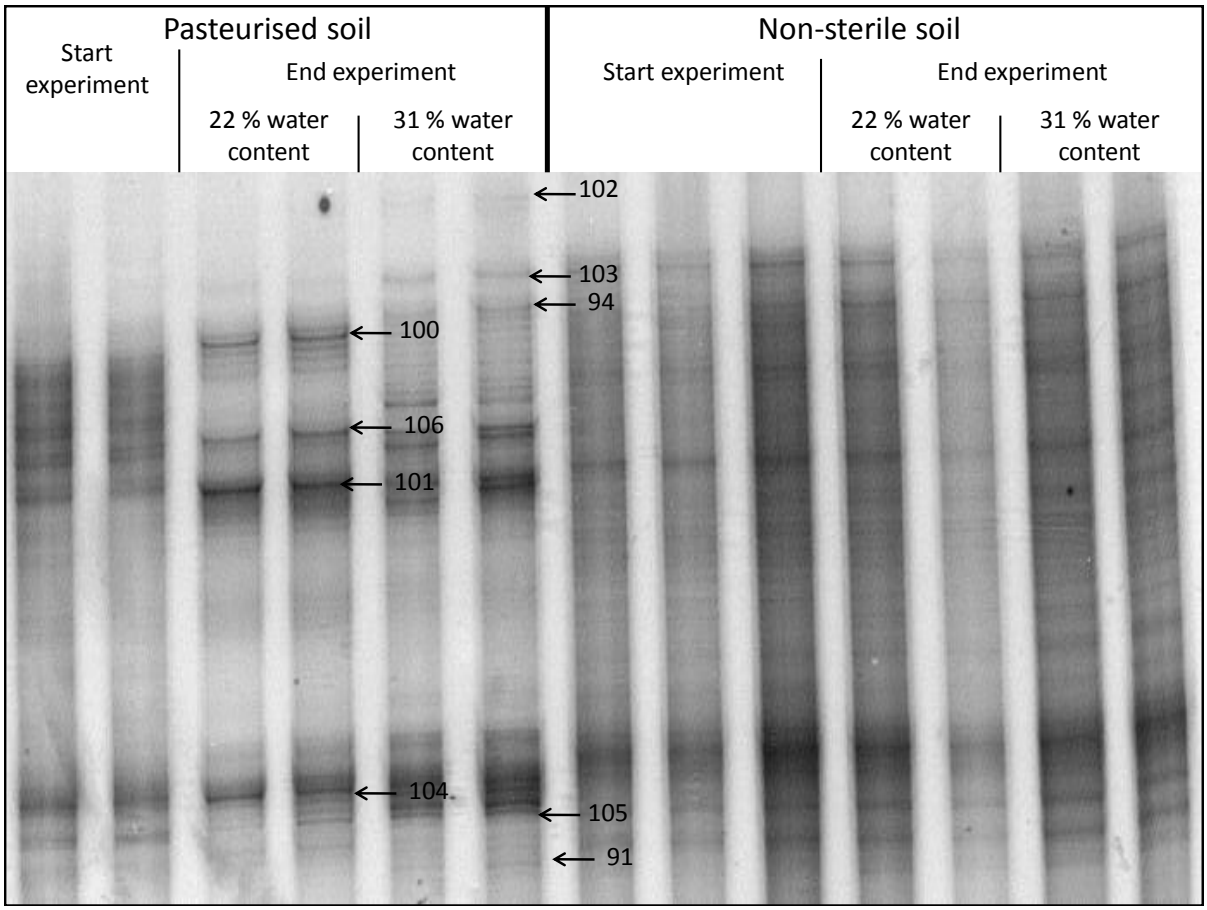

Figure 4.7 SSCP banding patterns of bacterial communities in pasteurised and non-sterile soils with different water content at the beginning and end of the experiment.

Table 4.5 Sequences matching of bacterial and fungal DNA extracted from SSCP gel

Band numbers	Sequence matches	Band numbers	Sequence matches
20, 21, 22, 23, 24	*Bacillus* spp. Cohn (Bacillales: Bacillaceae)	25, 40, 87, 91, 94, 102, 104, 106	uncultured bacterium sp.
30, 31 and 32	*Micromonospora* spp. Orskov (Actinomycetales: Micromonosporaceae)	28	uncultured *Herbaspirillum* sp. Baldani (Burkholderiales: Oxalobacteraceae)
34	order Actinomycetales	88	uncultured actinobacterium sp.
86	*Nonomuraea* sp. Nonomura (Actinobacteria: Actinomycetes)	41	uncultured *Verrucomicrobia* sp.
27, 29	*Herbaspirillum* sp.	97, 98	*Trichoderma* sp. Pers. (Ascomycota: Hypocreales)
42, 81	*Humicola* sp. Traaen (Ascomycota: Sordariales)	80, 75	uncultured Basidiomycota sp. Whittaher ex Moore
77, 78	*Sordariaceae* sp. Winter (Ascomycota: Sordariales)	79	uncultured Ascomycota sp.
67, 68, 99	*Geomyces* sp. Traaen (Ascomycota: Leotiomycetes)	100, 101, 103, 105	Sequence results ambiguous

The SSCP banding patterns of fungi recovered from pasteurised soil with different water content was similar between the beginning and end of the experiment and between the different water contents apart for two bands present in pasteurised soil at 22 % GWC at the end of the experiment which were not present in soil at 31 % GWC such as bands 67 and 68 (Fig. 4.8 and Table 4.5).

There was no difference in fungal banding patterns in non-sterile soil with different soil water contents between different treatments and time (Fig. 4.8).

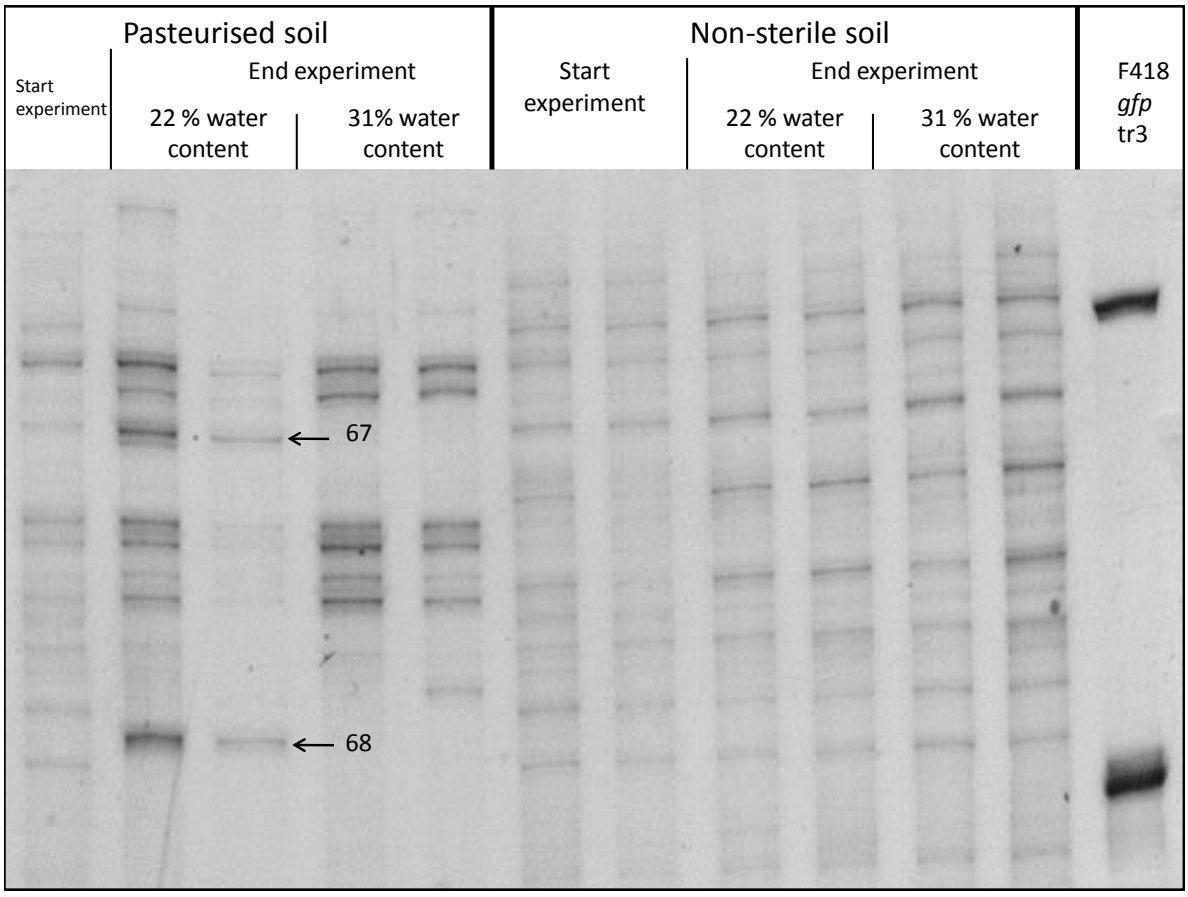

Figure 4.8 SSCP banding patterns of fungal communities in pasteurised and non-sterile soils with different water content at the beginning and end of the experiment. The arrows represent the sequenced DNA fragment.

4.3.3.2 Bacterial and fungal community changes in soil at different water potentials

As in the experiment with different water content, bacterial banding patterns changed from the beginning to the end of the experiment in pasteurised soil at different water potential (Fig 4.9 and Table 4.5). Some bacterial bands present in pasteurised soil at the beginning of the experiment were not present at the end, for example bands 20, 21, 22 and 24. Some bands present in pasteurised soil at -5 bar were not present at -0.33 bar, for example bands 30, 31, 32, 34, 86 and 87. Some bands present in pasteurised soil at -0.33 bar were not present at -5 bar such as bands 25, 28, 29 and 88. Band 27 was found in both pasteurised soil at -0.33 and -5 bar at the end of the experiment. Band 23, was found in all treatments and time in pasteurised soil. Band 30, 31 and 32 were in the same position than band 100 and surrounding bands in pasteurised soil at 22 % GWC (Fig. 4.7) and all sequences matched *Micromonospora* sp. Bands at the same location as bands 28 and 29 could be

observed in pasteurised soil at 31 % GWC (Fig 4.7), however the bands were not excised and sequenced.

There was no difference in bacterial banding patterns in non-sterile soil with different soil water contents between different treatments and time (Fig. 4.9 and Table 4.5). The number of bacterial bands was much greater in non-sterile soil than in pasteurised soil.

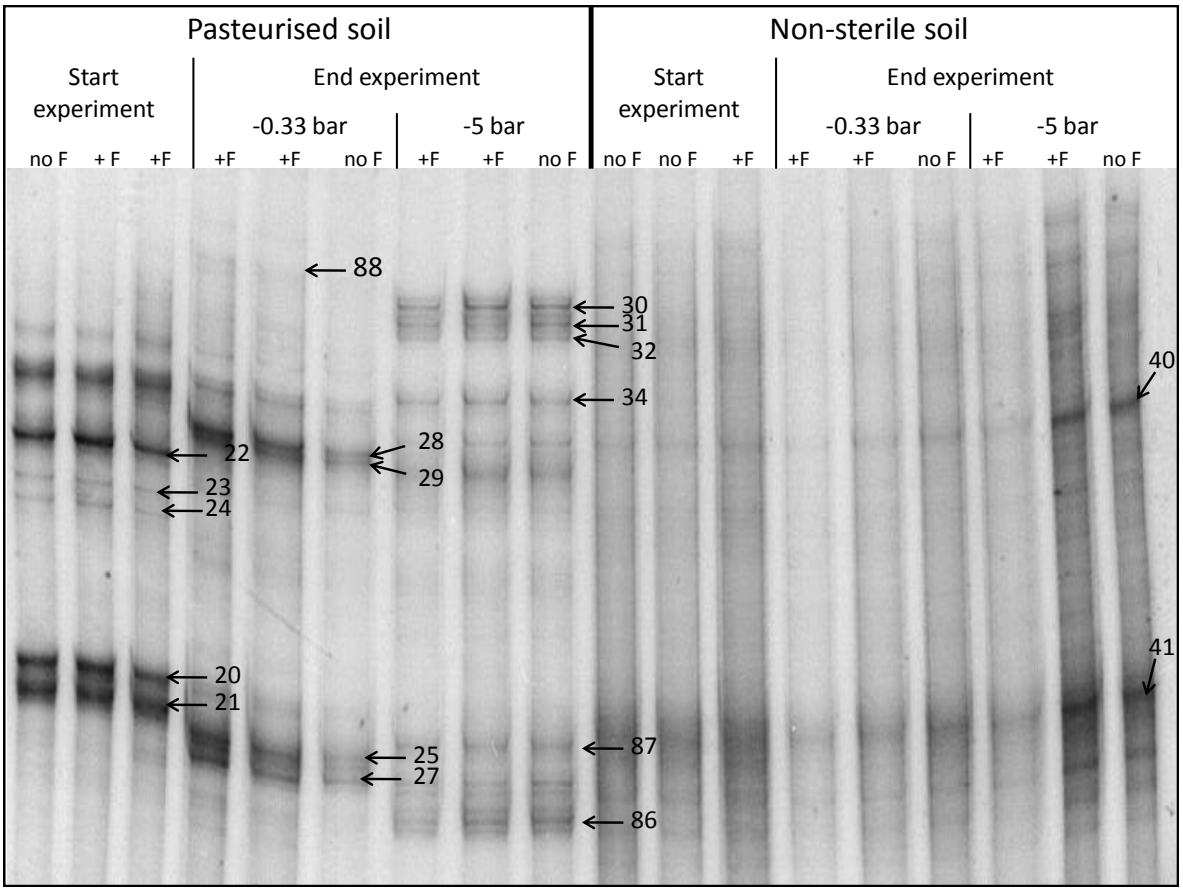

Figure 4.9 SSCP banding patterns of bacterial communities in pasteurised and non-sterile soils with different water potential at the beginning and end of the experiment. The arrows represent the sequenced DNA fragment from the gel slice.

The banding patterns representing the fungal communities in pasteurised soils showed a small change from the beginning to the end of the experiment (Fig. 4.10 and Table 4.5). At the end of the experiment, the fungal banding patterns in pasteurised soil at -0.33 and -0.5 bar were similar. Some fungal bands were common for both treatments at the end of the experiment in pasteurised soil, such as bands 97, 98 and band 99. Bands corresponding to F418 *gfp* tr3 were easily identified in pasteurised soil at -5 and -0.33 bar at the beginning and the end of the experiment. Band 99 was in the same position as band 68 in pasteurised

soil at 22 % GWC (Fig. 4.8) and both sequences matched *Geomyces* sp. Traaen (Ascomycota: Leotiomycetes).

There was no difference in fungal banding patterns in non-sterile soil with different soil water contents between different treatments and time (Fig. 4.10). The number of fungal bands was much greater in non-sterile soil than in pasteurised soil. Bands corresponding to F418 *gfp* tr3 could not be observed in non-sterile soil at -5 and -0.33 bar at the beginning and the end of the experiment.

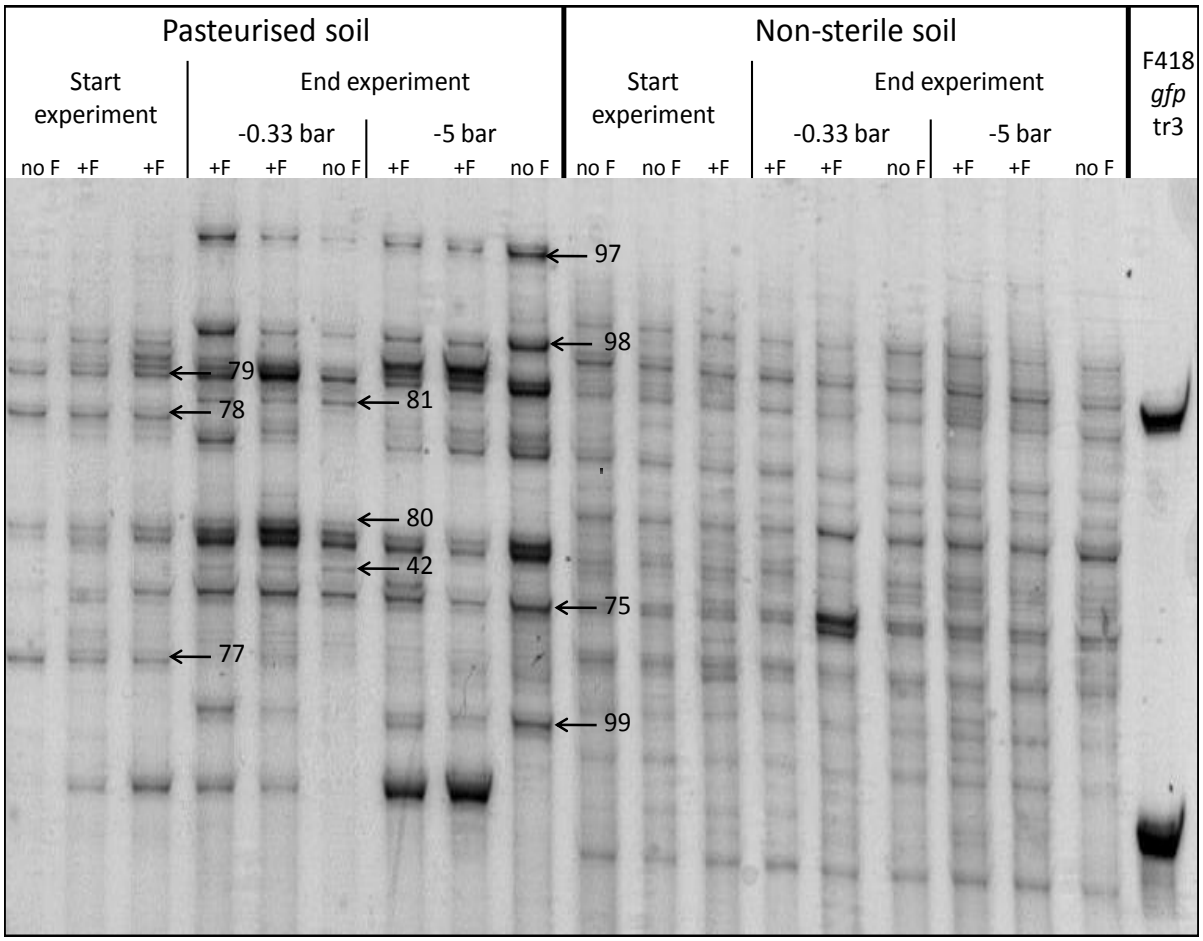

Figure 4.10 SSCP banding patterns of fungal communities in pasteurised and non-sterile soils with different water potential at the beginning and end of the experiment. The arrows represent the sequenced DNA fragment from the gel slice.

4.3.3.3 DNA concentration of soil microorganisms population

The concentration of the DNA extracted from soils samples at the beginning and the end of each experiment was measured using spectrophotometry and the results are given in Table

4.6 below. As expected, the DNA concentrations from pasteurised soils were significantly lower than from non-sterile soils at the beginning of the experiment with 3.38 and 13.72 ng/µL of DNA respectively. Similarly, at the end of the experiment, DNA concentrations from pasteurised soils were significantly lower than from non-sterile soils. There was no difference in DNA concentrations from non-sterile soils with low or high moisture level at the end of the experiment, whereas there was a significant difference in DNA concentrations from pasteurised soils with low or high moisture level at the end of the experiment. DNA concentrations from pasteurised soils with high moisture level were 1.22 ng/µL which was significantly lower than from pasteurised soil with low moisture level at 3.47 ng/µL at the end of the experiment.

Table 4.6 DNA concentration extracted from 0.25 g of soil eluted in 100 µL of elution solution at the start and end of the experiments mointoring the persistence of F418 *gfp* tr3 in soil at different water content and potential.

Soil treatment and moisture level		DNA concentration (ng/µL)			
		Start experiment		End experiment	
		Real value	Log10	Real value	Log10
Pasteurised soil	Low moisture	3.38	**0.32**	3.47	**0.459**
	High moisture	3.38	**0.32**	1.22	**-0.158**
Non-sterile soil	Low moisture	13.72	**1.13**	12.89	**1.104**
	High moisture	13.72	**1.13**	12.16	**1.076**
LSD (P < 0.05)			**0.496**		**0.465**

4.3.4 Effect of low and high moisture level on F418 *gfp* tr3 propagule dynamics in soil

4.3.4.1 Propagule recovery and mycelial growth in soil at different water content

The effect of soil water content on the number of propagules of F418 *gfp* tr3 washed from the membrane sandwich (0.2 µm pore) (referred as washed propagules) and on the mycelial growth in soil were assessed over 21 weeks (Table 4.7 and Fig. 4.11 and 4.12).

F418 *gfp* tr3 washed propagules in pasteurised soil at 31 % and 22 % GWC decreased significantly compared to the INIL during the first 2 weeks (Table 4.7 and Fig 4.11), while

mycelial growth appeared on all membranes in pasteurised soil from week 1 (Fig 4.12). F418 *gfp* tr3 washed propagules increased up to the INIL at 3 weeks in soil at 22 % GWC and at 7 weeks in soil at 31 % GWC and maintained that level until 10 weeks, followed by a significant decrease to 25 % of the INIL in soil at 31 % GWC and 47 % of the INIL in soil at 22 % GWC at 21 weeks. F418 *gfp* tr3 washed propagules in pasteurised soil at 22 % GWC were significantly higher than in pasteurised soil at 31 % GWC at 1 and 4 weeks, but it was not different at any other time.

Mycelial growth was present on all membranes in pasteurised soil at 22 and 31 % GWC at 1, 2, 3, 7 and 10 weeks. Mycelial growth was present on all membranes in pasteurised soil at 31 % GWC at 4 weeks while 75 % of the membranes in pasteurised soil at 22 % GWC had growth. Mycelial growth was present on all membranes in pasteurised soil at 22 % GWC at 21 weeks while 25 % of the membranes in pasteurised soil at 31 % GWC had growth.

F418 *gfp* tr3 washed propagules in non-sterile soil at 31 % and 22 % GWC decreased significantly compared to the INIL during the first two weeks (Table 4.7 and Fig 4.11), while mycelial growth appeared on only 25 % of the membranes at 22 % GWC (Fig 4.12). F418 *gfp* tr3 washed propagules in non-sterile soil at 31 % increased up to the INIL between 2 and 7 weeks. F418 *gfp* tr3 washed propagules from membranes in non-sterile soil at 22 % increased to the INIL at 7 weeks. F418 *gfp* tr3 washed propagules from membranes in non-sterile soil at 31 and 22 % GWC decreased significantly at 10 and 21 weeks and were 14 % of the INIL in soil at 31 % GWC and 2 % of the INIL in soil at 22 % GWC. F418 *gfp* tr3 washed propagules in non-sterile soil at 22 % was significantly lower than at 31 % GWC at 21 weeks.

Mycelial rings could be observed on only 25 % of the membranes in non-sterile soil at 22 % GWC at 1, 4, 7, 10 and 21 weeks. The first mycelial ring on membranes in non-sterile soil at 31 % GWC could be observed at 7 weeks on 50 % of the membranes, followed by 75 % of the membrane with a mycelial growth at 10 weeks and 50 % of the membranes at 21 weeks.

Mycelial growth diameters were not different between treatments or between times (Appendix J).

Table 4.7 F418 *gfp* tr3 washed propagules (CFU/sandwich and log_{10} value) from membrane sandwiches in pasteurised and non-sterile soil at 22 and 31 % GWC held at 15 °C over 21 weeks.

CFU/sandwich **(log_{10})**	Week 0	Week 1	Week 2	Week 3	Week 4	Week 7	Week 10	Week 21
Pasteurised soil at 31 % GWC	1.00 × 10^5 **(6.00)**	2.14 × 10^5 **(5.33)**	2.81 × 10^5 **(5.45)**	3.98 × 10^5 **(5.60)**	3.32 × 10^5 **(5.52)**	7.41 × 10^5 **(5.87)**	7.93 × 10^5 **(5.90)**	2.55 × 10^5 **(5.41)**
Pasteurised soil at 22 % GWC	1.00 × 10^6 **(6.00)**	5.48 × 10^5 **(5.74)**	3.63 × 10^5 **(5.56)**	7.85 × 10^5 **(5.90)**	7.66 × 10^5 **(5.88)**	6.00 × 10^5 **(5.78)**	6.90 × 10^5 **(5.84)**	4.74 × 10^5 **(5.68)**
Non-sterile soil at 31 % GWC	1.00 × 10^6 **(6.00)**	3.47 × 10^5 **(5.54)**	5.31 × 10^5 **(5.73)**	5.61 × 10^5 **(5.75)**	6.10 × 10^5 **(5.79)**	7.26 × 10^5 **(5.86)**	1.76 × 10^5 **(5.25)**	1.35 × 10^5 **(5.13)**
Non-sterile soil at 22 % GWC	1.00 × 10^6 **(6.00)**	5.14 × 10^5 **(5.71)**	4.58 × 10^5 **(5.66)**	3.95 × 10^5 **(5.60)**	4.43 × 10^5 **(5.65)**	6.81 × 10^5 **(5.83)**	4.06 × 10^5 **(5.61)**	2.26 × 10^4 **(4.36)**
LSD (P < 0.05)				**0.30**				

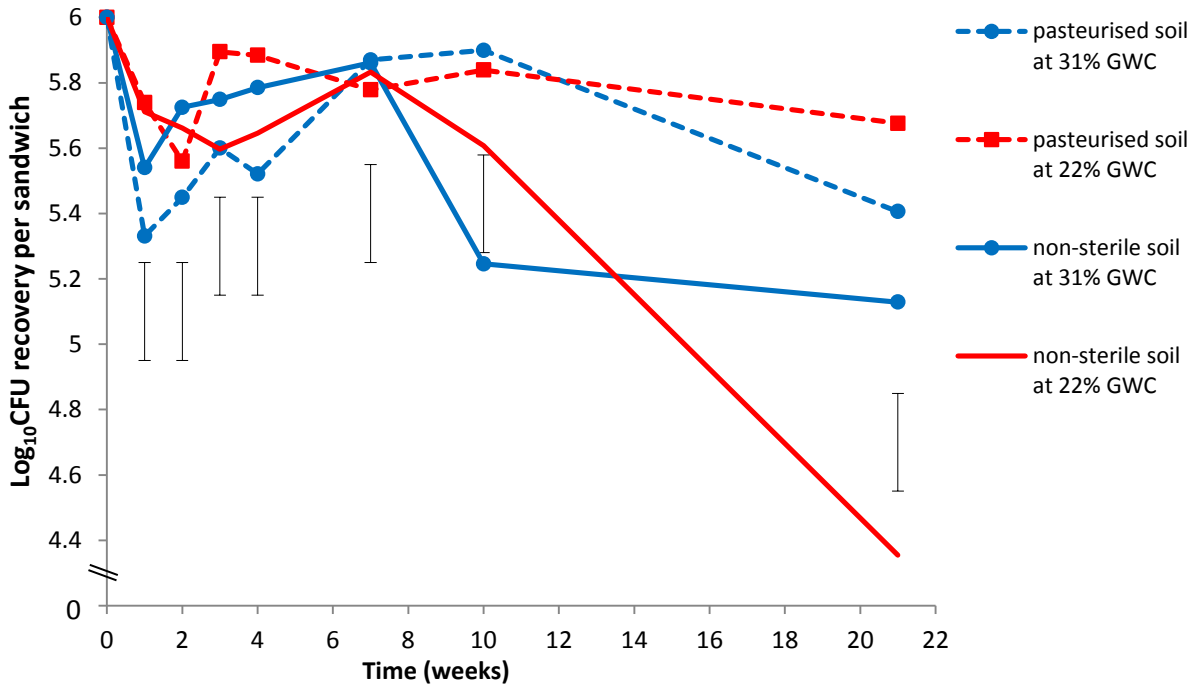

Figure 4.11 F418 *gfp* tr3 CFU recovered (log_{10}) from washed membranes sandwich (0.20 µm) in pasteurised and non-sterile soil at 22 and 31 % GWC held at 15 °C over 21 weeks. Vertical bars are LSD (P < 0.05).

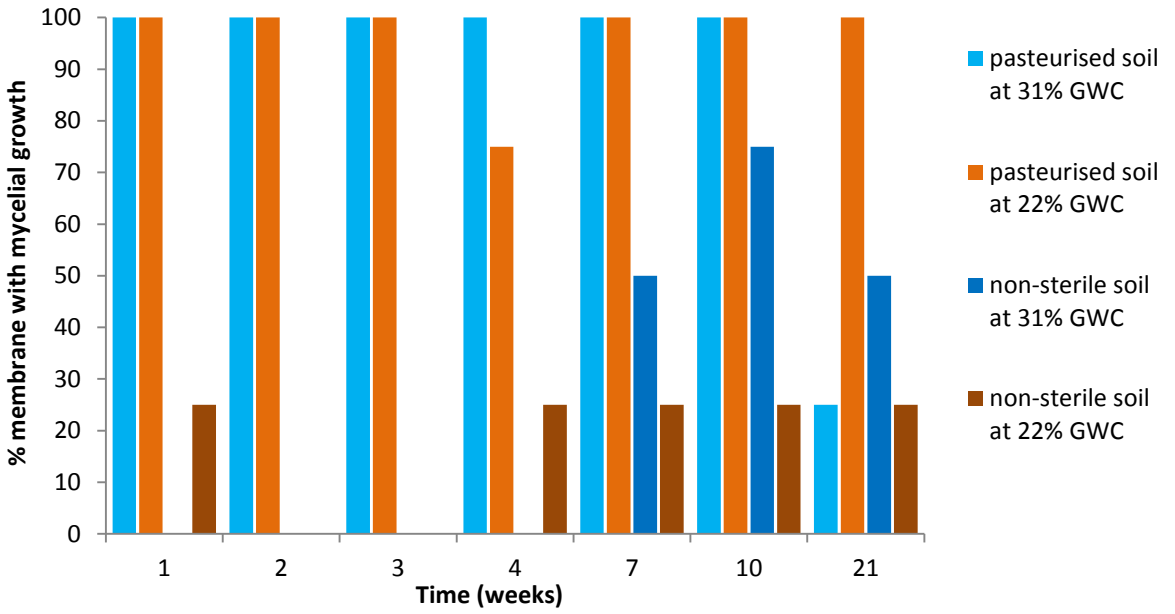

Figure 4.12 Percentage (of 4 replicates) of observed F418 *gfp* tr3 mycelial growth on membrane sandwiches in pasteurised and non-sterile soil at 22 % and 31 % GWC held at 15 °C over 21 weeks.

4.3.4.2 Propagule recovery and mycelial growth from membranes in soil with different water potential

The effect of soil water potential on the F418 *gfp* tr3 washed propagules (0.45 µm pore) and on the mycelial growth in soil were assessed over 20 weeks (Table 4.8 and Fig 4.13 and 4.14). F418 *gfp* tr3 washed propagules in pasteurised soil at -0.33 and -5 bar decreased significantly at 1 and 2 week respectively (Fig 4.13), while mycelial growth could be observed on all membranes (Fig 4.14). F418 *gfp* tr3 washed propagules in pasteurised soil at -0.33 bar were significantly lower than the INIL between 1 and 20 weeks and were 7 % of the INIL at 20 weeks. F418 *gfp* tr3 washed propagules in pasteurised soil at -5 bar were significantly lower than the INIL at 2, 8, 12, 16 and 20 weeks and was 35 % of the INIL at 20 weeks.

Washed propagule in pasteurised soil at -0.33 bar were significantly lower than in pasteurised soil at -5 bar at 1, 4, 8, 16 and 20 weeks. Mycelial growth could be observed on all membranes in pasteurised soil at both water potentials between 1 and 20 weeks except at 16 weeks where 75 % of the membranes at both water potentials showed a mycelial growth.

F418 *gfp* tr3 washed propagules in non-sterile soil at -0.33 bar were not different from the INIL between 0 and 12 weeks and decreased significantly at 16 and 20 weeks with a final recovery of 16 % of the INIL. While the washed propagules decreased at 16 and 20 weeks, mycelial growth appeared on 50 % and 75 % of the membranes respectively. F418 *gfp* tr3 washed propagules in non-sterile soil at -5 bar showed a slight decreased between 0 and 16 weeks although it was not significantly lower than the INIL and the final recovery was 60 % of the INIL. Mycelial growth could be observed on 25 % of the membranes at 4, 8, 12 and 16 weeks and was present on 50 % of the membranes at 20 weeks. There was no significant difference in washed propagules in non-sterile soil at -0.33 and -5 bar between 0 and 16 weeks and at 20 weeks it was significantly lower at -0.33 bar.

Mycelial growth diameters were not different between treatments or between times (Appendix J).

Table 4.8 F418 *gfp* tr3 washed propagule (CFU/sandwich and $\log_{10}$ value) from membrane sandwich in pasteurised and non-sterile soil at -0.33 and -5 bar held at 15 °C over 20 weeks.

CFU/sandwich **($\log_{10}$)**	Week 0	Week 1	Week 2	Week 4	Week 8	Week 12	Week 16	Week 20
Pasteurised soil at -0.33 bar	8.83×10^5 **(5.95)**	1.36×10^5 **(5.13)**	2.86×10^5 **(5.46)**	1.57×10^5 **(5.20)**	1.61×10^5 **(5.21)**	2.62×10^5 **(5.42)**	5.37×10^4 **(4.73)**	5.89×10^4 **(4.77)**
Pasteurised soil at -5 bar	8.83×10^5 **(5.95)**	4.90×10^5 **(5.69)**	3.08×10^5 **(5.49)**	4.75×10^5 **(5.68)**	3.76×10^5 **(5.58)**	3.33×10^5 **(5.52)**	1.97×10^5 **(5.29)**	3.10×10^5 **(5.49)**
Non-sterile soil at -0.33 bar	8.83×10^5 **(5.95)**	9.77×10^5 **(5.99)**	9.95×10^5 **(6.00)**	8.69×10^5 **(5.94)**	8.02×10^5 **(5.90)**	7.52×10^5 **(5.88)**	3.30×10^5 **(5.52)**	1.44×10^5 **(5.16)**
Non-sterile soil at -5 bar	8.83×10^5 **(5.95)**	7.89×10^5 **(5.90)**	6.71×10^5 **(5.83)**	5.83×10^5 **(5.77)**	6.03×10^5 **(5.78)**	4.55×10^5 **(5.66)**	4.39×10^5 **(5.64)**	5.28×10^5 **(5.72)**
LSD ($P < 0.05$)				**0.32**				

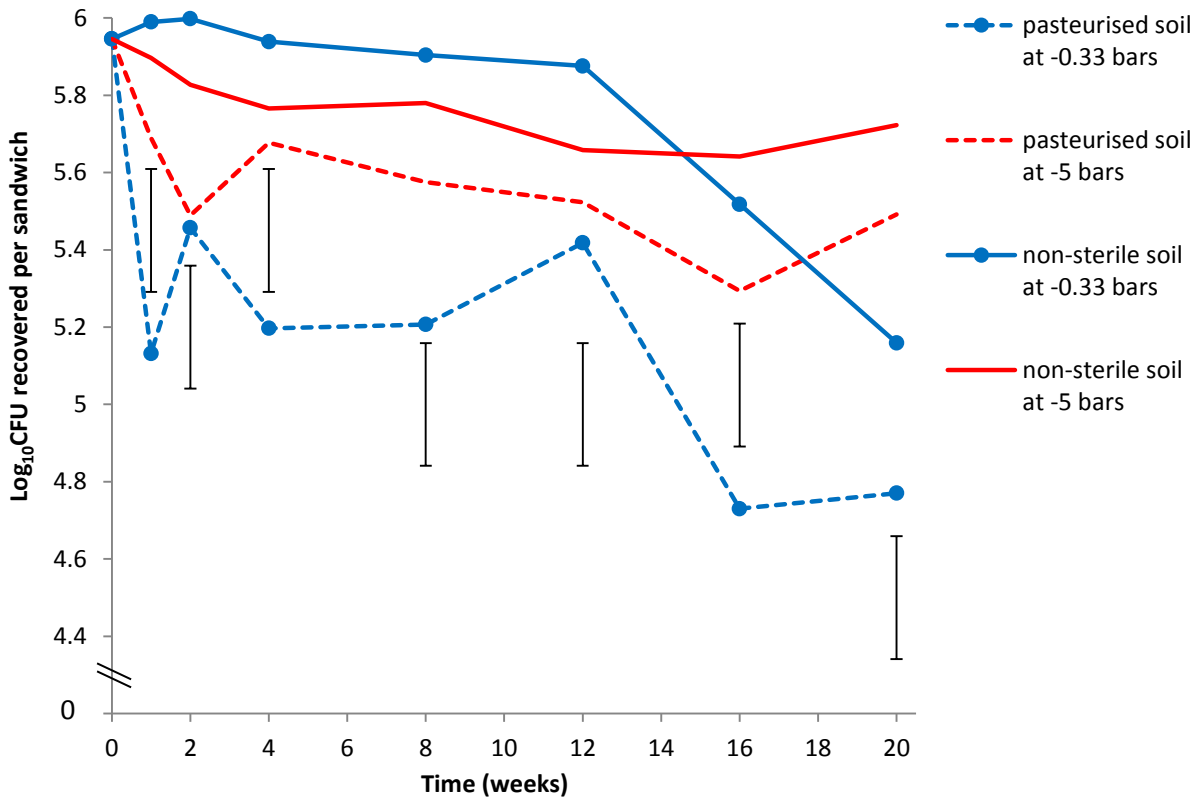

Figure 4.13 F418 *gfp* tr3 CFU recovered (log_{10}) from washed membranes sandwich (0.45 µm) in pasteurised and non-sterile soil at -0.33 and -5 bar at 15 °C over 20 weeks. Vertical bars are LSD ($P < 0.05$).

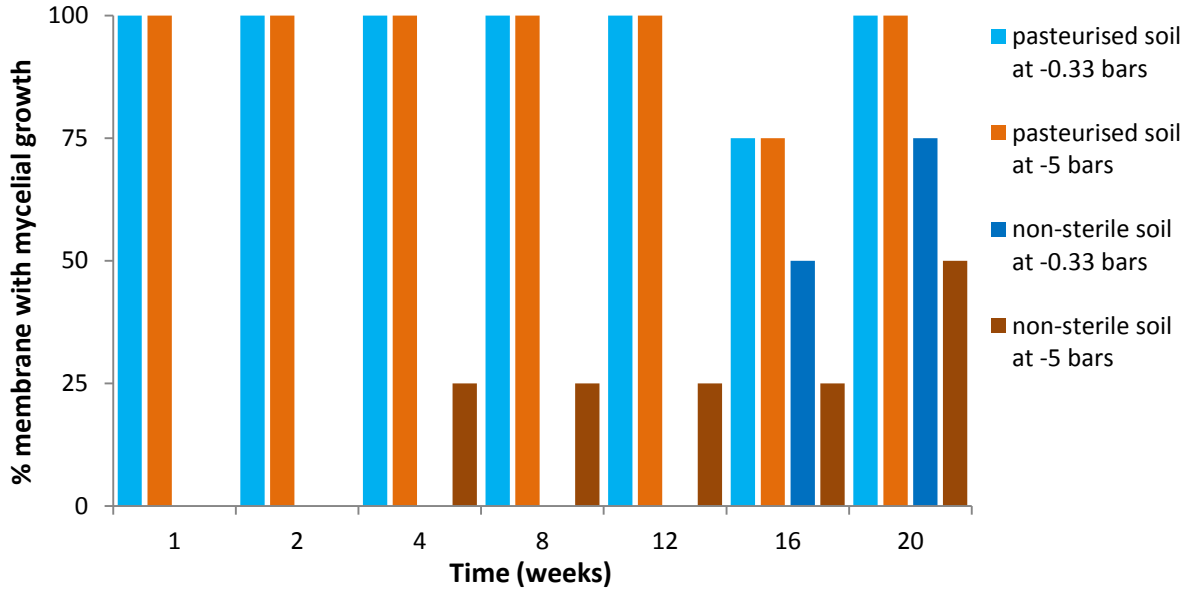

Figure 4.14 Percentage (of 4 replicates) of observed F418 *gfp* tr3 mycelial growth on membrane sandwiches in pasteurised and non-sterile soil at -0.33 and -5 bar at 15 °C over 20 weeks.

4.4 Discussion

4.4.1 Effect of soil moisture on F418 *gfp* tr3 populations

F418 *gfp* tr3 populations were significantly higher with low moisture level than with high moisture level in pasteurised soil. These findings are in accordance with results obtained in Chapter 3 with F418. These results are also similar to those obtained by Lingg & Donaldson (1981) and Quintela *et al.* (1982). Similarly to the results obtained in Chapter 3, F418 *gfp* tr3 populations in pasteurised soil at low moisture level increased significantly during the 2 initial weeks and were then maintained significantly higher than the INIL until the end of the experiments (maximum 18 weeks). F418 *gfp* tr3 populations in pasteurised soil at high moisture level were significantly lower than the INIL at the end of each experiment which was similar to results obtained in Chapter 3 and to results found by Quintela *et al*. (1992). F418 *gfp* tr3 populations in pasteurised soil followed the same trends in both experiments with different water content or water potential. The better persistence of F418 *gfp* tr3 in pasteurised soil at low moisture levels means that irrigation of pastures where F418 is applied may affect inoculum levels. Further research at field sites using a molecular assay that differentiates F418 from indigenous strains should be done to investigate persistence under field conditions. Once this information about timing and volume of irrigation is known farmers could be made aware of the potential loss of persistence in wetter soil when F418 is applied in high irrigation or rain fall regions.

Unlike Lingg & Donaldson (1981) and Quintela *et al.* (1982), F418 *gfp* tr3 populations in non-sterile soil at 15 °C were not different with low and high moisture level. This finding is, however, similar to results obtained by O'Callaghan *et al.* (2001) who found no difference in *B. bassiana* A6 CFU recovery in non-sterile soil at 23 and 30 % GWC during incubation at 15 °C for 123 d (approximately 18 weeks). Studdert *et al.* (1990) observed a difference in *B. bassiana* survival in soils at -0.3, -2 or -15 bar when the incubation temperature was 16 °C but they found no differences when the soil was held at 28 °C. The temperature used in their experiment (28 °C) was much higher than the present experiments held at 15 °C. Although the present results are different from Lingg & Donaldson (1981), Studdert *et al.* (1990) (experiment held at 16 °C) and Quintela *et al.* (1982), the experimental conditions were also different. Lingg & Donaldson (1981) studied the survival of *B. bassiana* in non-sterile soil at 25, 50 and 75 % saturation during 265 d (approximately 38 weeks) which was

considerably longer than the present experiments. Also, they used percentage saturation to describe their soil moisture levels which is difficult to compare to the present soil moisture levels measured using gravimetric soil moisture content and water potential. Studdert *et al.* (1990) studied *B. bassiana* survival in non-sterile soil at -0.3, -2 and -15 bar for up to 50 weeks, which is also significantly longer than the present experiment. The present experiment time length (maximum 18 weeks) was similar to O'Callaghan *et al*. (2001) who observed the same findings. It is possible that the absence of difference in F418 *gfp* tr3 persistence in non-sterile soil at low and high moisture is due to the shorter duration of the experiment (Lingg & Donaldson, 1981; Studdert *et al*., 1990). Quintela *et al.* (1992) found a difference in *B. bassiana* recovery in non-sterile soil held at 25 and 75 % saturation for 129 d (approximately 18 weeks) at 17 °C. Their timeframe was similar to the present timeframe of 18 weeks but they used percentage saturation to describe their soil moisture and 75 % saturation is likely to be wetter than the present high moisture levels (-0.33 bar and 31 % GWC). It is likely that the timeframe of 18 weeks used in the present experiment was not long enough to show differences between the treatments.

F418 *gfp* tr3 CFU recovery was consistent in both experiments in non-sterile with either different water content or potential. F418 *gfp* tr3 populations in non-sterile soil with low or high moisture levels showed a slow decrease over time which was significantly lower than the INIL at the end of both experiments. Previous studies (Lingg & Donaldson, 1981; Studdert *et al.*, 1990; Quintela *et al.*, 1992) found a decrease of *B. bassiana* CFU recovery in non-sterile soil at different soil moisture levels over time at a similar temperature.

4.4.2 Impact of soil moisture on bacterial and fungal communities

Fungal and bacterial communities were affected by the soil treatment (pasteurised and non-sterile soil) and by the moisture level in pasteurised soil. Bacterial community banding patterns were clearly different in pasteurised soil at low moisture level (22 % GWC and -5 bar) compared to high moisture level (31 % GWC and -0.33 bar) at the end of the experiments and also at the end of the experiments compared to the start of the experiments in pasteurised soil. Fierer *et al*. (2003) observed that bacterial communities (using terminal restriction fragment length polymorphism (T-RFLP) analysis) in a non-sterile soil under oak trees were affected by drying and rewetting cycles. Bacterial communities differed depending on the number of drying and rewetting cycles (from -0.6 to -150 bar) applied to the soil or if the soil was maintained at a constant moisture level of -0.6 bar. But the present

study is dissimilar to Fierer *et al.* (2003) as the experiment was made in pasteurised soil at two constant soil moisture levels. There is no similar study of the effect of different soil moisture levels on the microbial communities in pasteurised soil (or sterile soil).

The present results show that microbial communities (mostly bacterial) recolonising the pasteurised soils were different at low and high moisture levels. The pasteurised soil had a major part of its microbial population destroyed as the DNA concentration results showed, the quantity of DNA extracted from pasteurised soil was significantly lower than from non-sterile soil. The pasteurised soil had therefore empty ecological niches available for new microorganisms to develop in and it seems that the microbial recolonisation was influenced by the moisture level applied to the soil.

The two experiments using different water contents or water potentials had similar patterns of fungal and bacterial communities depending on the moisture applied. In both experiments, there were bands identified as *Micromonospora* sp. in pasteurised soil at low moisture level (22 % GWC or -5 bar). All sequenced bands in pasteurised soil at the start of the experiment with different water potentials were members of the genus *Bacillus*, probably because, as spore-formers, they have a high resistance to pasteurisation (Rodríguez-Lozano *et al*., 2010). Katzneslon (1940) showed that CFU recovery of diverse bacteria and fungi were affected by their combinations with other microorganisms. When *Azotobacter chroococcum* (Pseudomonadales: Azotobacteraceae), *Pseudomonas fluorescens* (Flugge) Migula (Pseudomonadales: Pseudomonadaceae), *Bacillus megatherium* (Bacillales: Bacillaceae), *Cunninghamella* sp. Matr. (Zygomycota: Mucorales) and *Humicola* sp. were combined together in sterile soil, their CFU recovery was affected by the combinations compared to their own recovery as single microorganisms in sterile soil. Studdert *et al*. (1990) suggested that lower *B. bassiana* conidia survival in high soil moisture levels was due to the effect of microbial population present and Lingg & Donaldson (1981) found that *P. urticae* was an inhibitor to *B. bassiana* in soil. SSCP results showed that there was a difference in microbial populations in pasteurised soil at low and high moisture and this difference in microbial populations is likely to contribute to the difference in F418 *gfp* tr3 populations observed in both experiments. The microorganisms present in pasteurised soil at high moisture level may be antagonistic to F418 *gfp* tr3. Some bacteria and fungi were successfully sequenced from the pasteurised soil at high moisture but the majority of the sequences did not match a specific genus and for the few identified species (i.e. *Herbaspirillum* sp. and *Humicola* sp.) there is no publication about their possible antagonistic effect on *B. bassiana*. Recovery of these species and iterative testing on *B. bassiana* cultures may indicate whether they are antagonistic.

In non-sterile soil there was no difference in bacterial and fungal community patterns at the end of the experiments at low or high moisture level and with the start of the experiments. These results are in accordance with published studies (Wu *et al.*, 2010; Fierer *et al*., 2003). Wu *et al.* (2010) found no difference in soil microbial communities using phospholipid fatty acids analysis (PLFA) in non-sterile soil incubated at 40, 60 and 80 % water holding capacity. Fierer *et al.* (2003) observed no differences in soil microbial communities (using T-RFLP analysis) in non-sterile grassland soil under different number of drying and rewetting cycles or under constant moisture. However, Fierer *et al.* (2003) used drying and rewetting cycles which is different from constant moisture levels used in the present study. The patterns of bacterial and fungal bands in non-sterile soil were consistent between the two experiments (water content and water potential). Microbial niches were already occupied in non-sterile soil; therefore there was little opportunity for competition and differential colonisation. Studdert *et al*. (1990) suggested that soil microbial population had an effect on *B. bassiana* conidia survival in non-sterile soil. In this study, there was no difference in soil microbial populations in non-sterile soil under different moisture levels and this correlated well with the absence of any difference in F418 *gfp* tr3 CFU recovery. This result shows that the impact of the soil microbial population on F418 *gfp* tr3 populations is probably more important than the direct impact of soil moisture level and a better understanding of the population dynamics of F418 in New Zealand pastures could be achieved with a thorough investigation of the variations in soil microbial populations with soil types, farming practices and seasons across New Zealand soils (Bardgett *et al*., 1999; Degens *et al*., 2000).

Lingg & Donaldson (1981) considered reduced O_2 and increased CO_2 in a high moisture level soil as a possible reason for the decrease in *B. bassiana*. Blair (1943) found that *Rhizoctonia solani* Kuhn (Basidiomycota: Cantharellales) growth was inhibited at high soil moisture level (i.e. 80 % soil saturation) due to poor aeration and increased in CO_2 in soil. There was no difference in microbial populations in non-sterile soil at low and high moisture level, therefore the hypothesis of a lack of O_2 and increase in CO_2 cannot be used in the present experiment. Blair (1943) observed an impact on the growth of *R. solani* at 50 and 80 % saturation, which were probably much wetter than the soil moisture used in the present experiment. The level of O_2 and CO_2 in the soils with different moisture levels used in the present experiment was most probably not a factor influencing the F418 *gfp* tr3 populations.

Bacterial and fungal communities were different in pasteurised and non-sterile soil. Bacterial and fungal communities banding patterns in non-sterile soil were higher in numbers than in pasteurised soil. The lower number of bands observed in pasteurised soil is due to the process of pasteurisation which reduced the total number of microorganisms in numbers and diversity. The quantity of DNA extracted from pasteurised soil was significantly lower than

from non-sterile soil even at the end of the experiment. Marschner & Rumberger (2004) observed a similar difference in band diversity between native soil and sterile soil while studying the recolonisation of sterile soil by wheat rhizosphere bacteria. In their experiment, the diversity of bands from sterile soil was significantly lower than from native soil. In the present study, the DNA concentration at the end of the experiment was significantly lower from pasteurised soil at high moisture level than at low moisture level. F418 *gfp* tr3 populations were significantly higher in pasteurised soil at low moisture level compared to high moisture level and the difference in DNA quantity might be due in part to the increase in F418 *gfp* tr3 populations in pasteurised soil at low moisture.

The SSCP technique provided a qualitative approach to assessing the soil microbial communities. Quantitative technique such as dilution plating on semi-selective media used by Shimazu *et al*. (2002) to estimate populations of bacteria, actynomycetes, fungi, and *B. bassiana* in soil could have been considered; however, since most soil microorganisms are not culturable on semi-selective media, this technique would underestimate the real quantity of each micro-organism. Molecular techniques such as SSCP and T-RFLP (Fierer *et al*., 2003) provide more information about the structure of the soil microbial communities and the changes within the communities since DNA of non-culturable microorganisms can be extracted.

4.4.3 F418 *gfp* tr3 dynamics in membrane sandwich in soil

The membrane sandwich experiment permitted the partial quantification of the different type of propagules (conidia or mycelia) in soil under different moisture levels, based on the observation that most conidia could be washed off the hydrophilic membrane (Holder and Keyhani, 2005) and that most mycelia would stay attached to the solid membrane substrate such as onto a solid agar plate. Holder & Keyhani (2005) observed that *B. bassiana* aerial conidia can be washed off hydrophilic surfaces easily. Therefore, the washed propagules were likely to be composed mainly of conidia. In other studies the soil sandwich method (Grose *et al*., 1984; Mekwatanakarn & Sivasithamparam, 1987; Simon *et al*., 1988; Wakelin *et al*., 1999) has only been used to measure mycelial growth, while the present study modified the soil sandwich method to assess different type of fungal propagules in soil.

The membrane sandwich experiments showed that there was a significant decrease of washed propagules (mainly conidia) at the same time as there was development of mycelia

on all membrane sandwiches held in pasteurised soil at both moisture levels during the first and second week in both experiments (water content and water potential). This experiment also demonstrated that washed propagules CFU increased after the first or second week in pasteurised soils but the increase never reached over the INIL. The increase of washed propagules is likely to be newly produced conidia. These findings indicate that some F418 *gfp* tr3 conidia germinated and developed as mycelia on the membranes held in pasteurised soil and that the mycelia subsequently produced a limited amount of conidia. A germinated conidium, probable piece of mycelium and a cluster of conidia could be observed under fluorescent microscopy in the pasteurised soil suspensions which corroborate the previous hypothesis. Pereira *et al*. (1993) observed that conidia of *B*. *bassiana* 447 could grow and sporulate in sterile soil. The authors placed 7.4 x 10^6 conidia in the centre of a Petri dish containing 15 g of sterile soil. Mycelial growth and sporulation could be observed 3 d post inoculation, and CFU counts showed an increase ranging from 7 fold of the initial inoculum in the soil amended with chitin to 140 fold in soil amended with ground fire ant tissue. Lingg & Donaldson (1981) and Quintela *et al*. (1992) observed some *B. bassiana* CFU increase in sterile soils and hypothesised that *B. bassiana* could grow in pasteurised soil, however they provided no direct proof. This experiment showed that the F418 *gfp* tr3 population increase that was observed in pasteurised soil at a low moisture level was probably due mainly to mycelia development in soil and not conidiation.

The washed propagules CFU recovery from membranes in pasteurised soil in both experiments were significantly lower than the INIL at the end of the experiments but there was a difference between the experiments with water contents and water potentials. In the experiment using water content measurement and the membrane with a pore size of 0.20 µm, there was no difference in washed propagules CFU recovery in pasteurised soil between the low and high moisture level, whereas, there was a significant difference in the experiment with water potential using the membrane with a pore size of 0.45 µm. The membrane with the pore size of 0.20 µm would not allow most microorganisms to access the interior of the membrane sandwich, whereas the 0.45 µm membranes would allow some microorganisms to access the interior of the membrane sandwich. Christensen *et al.* (1999) found that the major part of active bacteria in soil ranges in size between 0.25 and 0.50 µm. The environment inside the 0.20 µm membranes in pasteurised soil was likely to be artificially sterile at both the low and high moisture levels, while the environment inside the 0.45 µm membranes was likely to have different microbial populations as shown by the SSCP results. The different microbial populations at low and high moisture level and the effect of membrane pore size were likely to be responsible for the differences in washed propagules CFU recovery observed.

Washed propagules recoveries and mycelial ring frequency in non-sterile soil were different in the experiment using 0.20 µm membranes (with water content) and 0.45 µm membranes (water potential). As discussed above, the 0.20 µm membranes would not allow access to most microorganisms inside the membranes sandwich; therefore creating an artificially sterile environment. Results obtained using the 0.20 µm membrane were therefore not representative of the environmental conditions in the non-sterile soil surrounding the membranes compared to using 0.45 µm membrane.

The washed propagules CFU recoveries from 0.45 µm membranes in non-sterile soil were similar to the INIL until 12 weeks and mycelial rings were only evident in 25 % of the membranes at low moisture (-5 bar) at 4, 8 and 12 weeks. These results showed that F418 *gfp* tr3 populations were mostly maintained as conidia and did not produce mycelia (or in a very limited manner in soil at -5 bar) in non-sterile soil in the first 12 weeks. The presence of other microorganisms in non-sterile soil as shown by SSCP may have prevented germination of conidia by fungistasis. Dynamics of propagule types in non-sterile soil changed at low and high moisture level after 3 months. A significant decrease of washed propagules was observed in non-sterile soil at -0.33 bar at 16 and 20 weeks, concurrent to an increase of appearance of mycelial rings, whereas washed propagules recovery at -5 bar was not different from the INIL. These results showed that F418 *gfp* tr3 populations were mostly maintained as conidia in non-sterile soil at low moisture over 20 weeks whereas F418 *gfp* tr3 conidia started to germinate and produced mycelia after 3 month incubation. No difference in F418 *gfp* tr3 populations were observed in non-sterile soil at low and high moisture after 18 weeks, however, the propagule types might have been different as shown with the membrane sandwich experiment. A longer experiment such as done by Lingg and Donaldson (1981) and Quintela *et al.* (1992) might have shown a difference in F418 *gfp* tr3 populations in non-sterile soil at different moisture level.

There was no increase in the mycelial ring diameter in any of the treatments (Appendix J) showing that F418 *gfp* tr3 could have only a limited development as hyphae mass in soil, which is in accordance with the results of Walstad *et al.* (1970).

The use of modified membrane sandwiches in soil gave an interesting insight into population dynamics of F418 *gfp* tr3 in pasteurised and non-sterile soil at different moisture levels. The modified membrane sandwich method showed that F418 *gfp* tr3 populations increase observed in pasteurised soil is likely due to the formation of mycelia and that the better persistence of F418 *gfp* tr3 in pasteurised soil at low moisture is likely due to the higher number of conidia recovered. This method also showed that even though there was no difference in F418 *gfp* tr3 population level in non-sterile soil using the soil dilution plating

method, the ratio of conidia/mycelia is likely to be different over time and is likely to lead to a difference in F418 *gfp* tr3 persistence in non-sterile soil at low and high moisture levels. Although the fungus was not in direct contact with the soil, which is an obvious drawback, it still was a cost-effective and easy technique. The use of qPCR was planned but the length of time to optimise the assay and the cost of the technique meant that this more resource efficient method was used.

4.4.4 Pasteurisation and change in soil moisture release curve

The use of the pressure plates showed that the moisture release curves for pasteurised and non-sterile Templeton silt loam soil were significantly different. Between -0.5 and -10 bar, the water content of pasteurised soil was significantly lower than in non-sterile soil for the same water potential. The process of pasteurisation is likely to have changed the structure of the soil and changed the moisture holding capacity compared to non-sterile soil which did not receive any temperature treatment. Raveh & Avnimelech (1978) found that drying of the soil led to the destruction of the bonds between organic macro molecules and therefore decreased the stability of organic matter in soil. Although there was a significant difference in water availability between the experiment with water content and water potential (i.e. 22 % GWC is equal to -2.15 bar in non-sterile soil and -0.85 bar in pasteurised soil), the trends for the CFU recovery of F418 *gfp* tr3 in soil with different water content were the same for each soil type/moisture level treatment in soil with different water potential. The difference in water availability between the experiments using water content and water potential did not seem to affect the results obtained.

Soil moisture of the Templeton silt loam should have been described using percentage field capacity when water potential was not available (Jaronski, 2007) as the gravimetric water content does not give any information about the water availability. The field capacity of Templeton silt loam was measured at 31 mL H_2O/100 g of dry soil (New Zealand Soil Bureau, 1968) but the use of the pressure plates showed that the field capacity for non-sterile Templeton silt loam soil was 34.5 mL H_2O/100 g of dry soil. Therefore the experiments set up before the drawing of the moisture release curves using the pressure plates were expressed in the same gravimetric water content but the percentage of field capacities were different.

4.4.5 Conclusions

The following conclusions can be drawn from the results presented in this chapter:

- The pasteurisation process changed the soil moisture release curve of the soil; therefore only water potential should be used for soil experiments.
- F418 *gfp* tr3 persistence in pasteurised soil was similar to F418 persistence in pasteurised soil at low and high moisture observed in Chapte*r 3*. However, F418 *gfp* tr3 persistence in non-sterile soil was different.
- Fluorescent microscopy showed that different F418 *gfp* tr3 propagules types (conidia, germinated conidia, mycelia) were present in pasteurised soil. However, the observations were challenging and a brighter F418 *gfp* transformants would be needed for future experiments.
- The use of the SSCP analysis showed a correlation between changes in F418 *gfp* tr3 persistence and changes in soil microbial communities
- The modified membrane sandwich method used in this chapter and the SSCP analysis helped to understand F418 *gfp* tr3 propagules dynamics in soil.
- F418 *gfp* tr3 populations increase observed in pasteurised soil at low moisture level were probably mostly due to mycelia development.
- There were differences in propagule types in the non-sterile soil with different soil moisture levels after 3 months incubation.
- F418 *gfp* tr3 populations were higher in pasteurised soil at low moisture level and conidia density was more stable in non-sterile soil at low moisture level. Soil moisture may affect F418 population levels in the soil, therefore field experiments on irrigation/rainfall and its interaction with soil type should be done in the future.

Chapter 5
Beauveria bassiana F418 *gfp* tr3 persistence in soil in presence of host and non-host insects

5.1 Introduction

There are numerous studies on the interaction of entomopathogenic fungi with their host arthropod. However, publications on the interaction between entomopathogenic fungi and non-host arthropods in their soil environment are scarce. In Chapter 3, the presence of a non-host insect *C. zealandica* was shown to have a negative impact on *B. bassiana* F418 population in soil. Some authors have reported conidial attachment and germination on non-host arthropod cuticle (Boucias *et al.*, 1988; Sitch & Jackson, 1997; Hajek & Eastburn, 2003; Ment *et al*., 2012) and the loss of conidial viability via ingestion in non-host arthropod (Dromph, 2001) or via the presence of antagonistic bacteria and protozoa in soil (Fargues, 1983). Understanding the interaction between *B. bassiana* F418 *gfp* tr3 and *C. zealandica* is important as *C. zealandica* is a common pest in New Zealand pasture where *B. bassiana* F418 would potentially be applied for the control of *S. lepidus*.

Attachment and germination of *B. bassiana* F418 to *C. zealandica* cuticle leading to a loss of conidia was one of the hypotheses given to explain the decrease of *B. bassiana* F418 in presence of a non-host insect in Chapter 3. Boucias *et al.* (1988) found that conidia from entomopathogenic fungi *B. bassiana* and *M. anisopliae* could bind to the non-host ghost cuticle of velvetbean caterpillar *A. gemmatalis* larvae. Sitch & Jackson (1997) and Hajek & Eastburn (2003) observed that conidia of *L. lecanii* (formerly *Verticillium lecanii*) and *E. maimaiga* respectively could attach and germinate on non-host arthropod cuticle. Ment *et al.* (2012) observed that *gfp* transformed *Metarhizium* spp. could geminate and form appressoria on cuticle of non-host tick *H. excavatum* but could not penetrate the cuticle and died after a few days.There is no report of attachment and germination of *B. bassiana* conidia on *C. zealandica* cuticle.

Loss of *B. bassiana* F418 conidia through ingestion by *C. zealandica* larvae was another hypothesis given to explain the decrease of F418 in Chapter 3. Dromph (2001) found that ingestion of *M. anisopliae* conidia by collembolans species *F. fimetaria* and *P. minuta*

reduced the viability of conidia. Lacey *et al*. (1988) found digested conidia of *M. anisopliae* in the digestive system of host *C. quinquefasciatus*. There is no similar report for *B. bassiana* ingested by *C. zealandica*.

Presence of antagonistic bacteria and amoeba on *C. zealandica* (cuticle and guts) degrading F418 conidia was a third hypothesis that could explain the decrease of *B. bassiana* F418 in Chapter 3. Fargues *et al.* (1983) showed that some soil bacteria and amoeba can degrade *B. bassiana* blastospores.

The objectives of this chapter were first to confirm the findings made in Chapter 3 on the persistence of F418 in the presence of host and non-host insects in pasteurised soil using F418 *gfp* tr3 in pasteurised and non-sterile soil. A second objective was to examine the hypotheses suggested in Chapter 3 for the lower recovery of F418 in pasteurised soil with non-host insect (loss of conidia by germination on non-host cuticle or ingestion by non-host or presence of antagonists in soil). *Beauveria bassiana* F418 *gfp* tr3 will be referred as F418 *gfp* tr3 in this chapter.

5.2 Material and methods

5.2.1 Evaluation of F418 *gfp* tr3 persistence in soil with host, non-host and no insects

5.2.1.1 Host and non-host experimental procedure: Experiment 2009

The objective of this experiment was to determine if the persistence of F418 *gfp* tr3 in presence of host, non-host and without insects in pasteurised and non-sterile soil at 22 % GWC was similar to the persistence of F418 observed in pasteurised soil in the presence of host, non-host and without insects in Chapter 3.

The first experiment was set up in August 2009 using 60 mL screw cap containers (LabServ, New Zealand) filled with 15 g of dry pasteurised soil or equivalent amount of non-sterile soil and inoculated with 2 x 10^5 F418 *gfp* tr3 conidia/g DWS. F418 *gfp* tr3 conidia were produced and their viability checked as described in section 2.2.1.2. The pasteurised soil used was as described in section 3.2.1 and the non-sterile soil was collected in July 2009 and treated as

described in the section 3.2.1. Both pasteurised and non-sterile soils were moistened to 22 % GWC (Table 4.2). Field collection and treatment of non-host insect *C. zealandica* larvae was described in section 3.2.6.4.2. *Tenebrio molitor* 4-5th instar larvae (Biosuppliers, Auckland) were used as the host insect. Prior to adding the insects to the soil, *C. zealandica* and *T. molitor* larvae were starved for 48 h to partially empty their guts. *Costelytra zealandica* larvae in their late 3rd instar stage were washed in a 0.01 % Triton® X-100 (BDH, Poole, UK) suspension for 20 s by gentle hand mixing in a beaker, followed by rinsing in dH_2O and left on a filter paper for 2 min to remove excess water. The effect of presence of host and non-host insects on the persistence of F418 *gfp* tr3 in pasteurised and non-sterile soil was determined by the addition of one 4-5th instar larva of *T. molitor* as a host insect or one 3rd instar larva of *C. zealandica* as a non-host insect or no insects to both pasteurised and non-sterile soil. The *C. zealandica* larval density used was equivalent to nearly 300 larvea/m^2. There were four replicate containers per treatment per sampling time and the containers were incubated at 15 °C in the dark using a RCBD.

5.2.1.2 Host and non-host experimental procedure: Experiment 2010

The objective of this experiment was to determine if the persistence of F418 *gfp* tr3 in presence of host, non-host and without insects in pasteurised and non-sterile soil at -2 bar was similar to the persistence of F418 *gfp* tr3 with similar treatment at 22 % GWC as described in section 5.2.1.1.

The second experiment was set up in March 2010 and the experimental set up and sampling used was as described in section 5.2.1.1 apart from the following details: the non-sterile soil was collected in March 2010 and treated as described in the section 3.2.1. Both pasteurised and non-sterile soils were moistened to -2 bar (Table 4.2) using different volumes of water as pasteurised and non-sterile soils do not have the same moisture release curve. *Costelytra zealandica* larvae were washed by spraying with dH_2O, instead of 0.01 % Triton® X-100 which was used in 2009 the experiment, to attempt to remove the field soil present on their cuticle. Triton® X-100, which is a detergent, could have degraded the non-host insect cuticle and was therefore not used during the 2010 experiment. Two additional replicate containers were prepared for each soil/insect treatment with and without F418 *gfp* tr3 addition to be sampled at that start and the end of the experiment for soil bacterial and fungal communities' study (5.2.2).

5.2.1.3 Assessment of fungal persistence in soil

Containers were destructively sampled at 0, 1, 2, 4, 8 and 12 weeks in the 2009 experiment and an additional sampling at 16 weeks occurred in the 2010 experiment. Persistence was monitored using the soil dilution plating method derived from Davet & Rouxel (2000). Total soil from each container was mixed with 135 mL of 0.01 % SDWA in a 250 mL screw cap container (LabServ, New Zealand). The protocol for evaluation of persistence was as described in section 4.2.2.2. Persistence was expressed as the number of CFU recovered/g DWS.

5.2.1.4 Observation of soil suspensions using fluorescence microscopy

The objective of this experiment was to observe F418 *gfp* tr3 in soil suspensions to determine which propagule types were present in the soils with different treatments.

Soil suspensions (10^{-1} dilution) were observed by fluorescence microscopy to determine the propagule types present in the soil as described in section 4.2.2.4. At least 30 % of all containers of week 0, 1, 2 and 4 of the 2009 experiment were observed with a minimum of one replicate for each treatment observed. The technique was not used thereafter as it was considered too difficult to observe F418 *gfp* tr3 propagules.

5.2.1.5 Observation of non-host insect cuticle

The objective of this experiment was to observe *C. zealandica* cuticles after each soil sampling to determine if F418 *gfp* tr3 could attach and germinate on *C. zealandica* cuticles.

Costelytra zealandica larvae from all replicate containers in both experiments were collected during each sampling and kept individually in a 1.5 mL tube at -20 °C for later cuticle observation. Pieces of cuticles (approximately 3 × 3 mm) were excised using sterile scalpels on the day of observation and washed to remove non-attached conidia. Cuticles were washed by placing them into a 1.5 mL tube containing 1 mL of 0.01 % Triton® X-100 and vortexing the tube for approximately 15 s. This was repeated three times with the cuticles being transferred to a new tube containing 0.01 % Triton® X-100 each time. The fourth wash was done in 1 mL of SDW. The washed cuticle was mounted with a drop of SDW onto a microscope slide with a cover slip and observed under light and fluorescence microscopy (Olympus BX50, magnification × 400, excitation filter Olympus U-MWIB3, software Olympus

Olysia and DP2-BSW, camera Olympus DP12 and DP72, Olympus Corp., Tokyo, Japan). A piece of cuticle from at least one *C. zealandica* larva from each sampling time was observed.

5.2.1.6 Observation of host insects for conidiation level

The objective of this experiment was to observe *T. molitor* larvae cadavers to determine if there was a difference in fungal conidiation supported by the cadavers, function of the soil type and time.

During the 2010 experiment, all dead *T. molitor* larvae were observed for supporting fungal conidiation during the soil sampling. Conidiation on cadavers was described as none, light, medium or heavy. Dead insects with no mycelial growth or 0% conidiation had a conidiation level described as none. The conidiation level on dead *T. molitor* larvae showing mycelial growth and reaching approximately 30% of the maximum potential conidiation was described as light. The conidiation level on dead *T. molitor* larvae showing heavy mycelium growth and reaching approximately 60% of the maximum potential conidiation was described as medium. The conidiation level on dead *T. molitor* larvae reaching approximately 100% of the maximum potential conidiation was described as heavy.

5.2.2 Comparison of fungal and bacterial communities present in soil with host, non-host and no insects

The objective of this experiment was to determine the fungal and bacterial soil community patterns function of the soil type, the presence of insects and time to establish if a correlation existed between the soil communities and the soil type/insect presence and the F418 *gfp* tr3 persistence.

Soil containers prepared for the study of soil fungal and bacterial communities (5.2.1.2) were sampled at the start and the end of the experiment set up in March 2010 and the 0.5 g soil samples were kept in 1.2 mL sterile screw cap microcentrifuge tube and stored at -80 °C until DNA extraction. DNA extractions, amplifications and electrophoresis for SSCP analysis were all performed as described in section 4.2.3.

5.2.3 Evaluation of the interaction between F418 *gfp* tr3 conidia and *C. zealandica* larvae cuticle

The objective of this experiment was to determine if F418 *gfp* tr3 could attach and germinate on live and ghost cuticle of *C. zealandica* larvae.

F418 *gfp* tr3 conidia were produced as described in section 2.2.1.2. *Costelytra zealandica* larvae (3rd instar) were used as a non-host insect (section 3.2.6.4) for this experiment, either alive or the cuticle was removed and used as a ghost cuticle.

5.2.3.1 Ghost cuticle collection and infection

Ghost cuticles were collected from *C. zealandica* 3rd instar larvae killed by freezing (collection and treatment of *C. zealandica* larvae is described in section 3.2.6.4). Pieces of cuticles (approximately 3 × 3 mm) were excised using sterile scalpels and mounted on microscope slides and 1 µL of a 4.8×10^5 F418 *gfp* tr3 conidia mL^{-1} suspension was placed on the cuticle. Slides with cuticles were placed on a filter paper moistened with 0.5 mL of SDW in a 90 mm Ø Petri dish (LabServ, New Zealand) at room temperature (20 °C ± 2) for incubation. The Petri dishes were held in a closed plastic bag with moistened paper to maintain high humidity during the experiment. There were four cuticles for each sampling time. Cuticles were sampled at 1, 2 and 3 d post infection and washed before observation to remove non-attached conidia. The washing and observation of cuticles was as described in section 5.2.1.5.

5.2.3.2 Inoculation of live *C. zealandica* larvae and cuticle observation

Collection and treatment of *C. zealandica* 3rd instar larvae is described in section 3.2.6.4. Live *C. zealandica* larvae were infected using three different methods: immersion in a conidial bath, drop application on the abdomen and larvae placed on a contaminated filter.

For the immersion method, batches of four *C. zealandica* 3rd instar larvae were immersed for 20 s in a 5 mL suspension of 1×10^8 F418 *gfp* tr3 conidia.ml^{-1} (unless otherwise stated) and excess liquid was removed by vacuum filtration followed by 2 min incubation on a dry filter paper at room temperature. *Costelytra zealandica* larvae inoculation using the immersion method was repeated four times (August 2009, June 2010 (twice) and October 2010).

Inoculated larvae were held on a filter paper moistened with 0.5 mL of SDW in either a Petri dish at room temperature for all experiments (20 °C ± 2) or in a 15 °C incubator (June 2010 experiment). Petri dishes were held in a closed plastic bag with moistened paper.

For the drop method, 10 µL of 1 × 10^8 F418 *gfp* tr3 conidia/mL suspension was placed on the abdomen of *C. zealandica* 3rd instar larvae. Inoculated larvae were placed in individual wells of a 6 well cell culture plate. The cell culture plates were maintained at either room temperature (20 °C ± 2) (all experiments) or in a 15 °C incubator (June 2010 experiment) in a closed plastic bag and covered with moistened paper.

For the contaminated filter method, 1 mL of 1 × 10^8 F418 *gfp* tr3 conidia/mL suspension was sprayed onto a filter paper placed in a Petri dish. Two *C. zealandica* 3rd instar larvae were kept per Petri dish. Petri dishes were held at room temperature (20 °C ± 2) in a closed plastic bag with moistened paper.

There were 10 larvae inoculated for each infection method. Larvae were sampled at 1, 2 and 3 d and kept at -20 °C until observation. Cuticles were removed, washed and observed using the protocol described in section 5.2.1.5.

5.2.4 Evaluation of F418 *gfp* tr3 conidia recovery after passage through *C. zealandica* larvae guts

The objective of this experiment was to determine if F418 *gfp* tr3 conidia viability was affected by digestion in *C. zealandica* guts.

5.2.4.1 *Costelytra zealandica* larval mouth injection with F418 *gfp* tr3 conidia

F418 *gfp* tr3 was used in this study and conidia were produced using the protocol described in 2.2.1.2. *Costelytra zealandica* 3rd instar larvae were used as a non-host insect. A 4.8 × 10^5 conidia/mL suspension of F418 *gfp* tr3 conidia was prepared in sterile 0.01 % Triton® X-100 and 5 µL of this suspension (corresponding to approximately 2,400 conidia) was injected into the mouth of a larvae using a micro-applicator (Burkard Arnold, type LV 65, Rickmansworth, UK) (Fig 5.1). Immediately following the mouth injection, a further 5 µL was placed in a 1.5 mL tube containing 495 µL of sterile 0.01 % Triton® X-100. These 5 µL droplets were used to measure the quantity of conidia injected in the larva's mouth as the conidia suspension

could not be kept homogenised inside the syringe. This 500 µL suspension was vortexed and diluted to 1/10 and a 100 µL of this dilution was plated onto 10 % SDA+A+Hyg. CFUs were counted after 14 d incubation at 20 °C in the dark and these CFUs counts were used to obtain an estimate of the number of conidia injected into each insect. This experiment was repeated three times (August 2009, April 2010 and May 2010) and 6 larvae were successfully force fed in August 2009, 37 in April 2010 and 48 in May 2010.

5.2.4.2 Method to measure F418 *gfp* tr3 conidia recovery after ingestion by *C. zealandica* larvae

After the mouth injection, larvae were held individually in 24 well plates at room temperature (20 °C ± 2) and were fed a piece of carrot (approximately 2 × 2 mm) daily. The well plates were kept in a closed plastic bag with moistened paper to keep humidity high. Faeces were collected daily for 9 to 11 d by pipetting 500 µL of sterile 0.01 % Triton® X-100 onto each larva in order to try to wash off the faeces in which the larvae were covered. Larvae were placed in a new clean well and fed a new piece of carrot. The faeces suspension was pipetted inside the well until all the faeces were mixed in the suspension. Faeces suspension (without dilution and at 10^{-1} dilution) was plated onto 10 % SDA+A+Hyg. There were duplicate plates per faecal sample. CFU were counted after 14 d incubation at 20 °C in the dark and the total number of conidia viable after digestion was estimated from the sum of the CFU recovered daily. The percentage of conidia recovered was assessed by dividing the total CFU recovered from faeces for each larva by the total conidia (in CFU) injected in each larva.

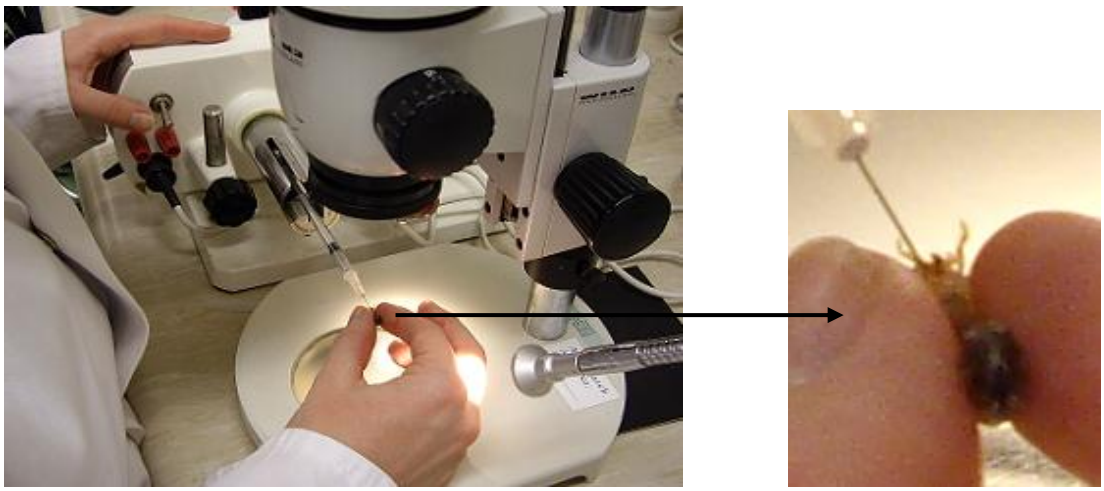

Figure 5.1 Mouth injection of F418 *gfp* tr3 conidia suspension into *C. zealandica* larvae using a micro-applicator.

5.2.4.3 *Costelytra zealandica* larvae cuticle observation post conidia ingestion

The objective of this experiment was to determine if F418 *gfp* tr3 conidia could be observed, attached or germinated, on the cuticle of *C. zealandica* using microscopy. At the end of the ingestion experiment, larvae were kept at -20 °C for later cuticle observation. At the time of observation, cuticles were removed, washed and observed for conidial attachment using the protocol described in section 5.2.1.5.

5.2.5 Statistical analysis

All CFU recovery data were $\log_{10}$ transformed to achieve a normal distribution essential to the use of a parametric test such as ANOVA (Olsen, 2003). A split plot ANOVA was carried out, with treatment factors being the treatments (soil type and insect presence) and time, with a linear polynomial contrast specified where appropriate. Significance was evaluated at $P < 0.05$ for all analyses and mean separation was executed by the unprotected LSD test. All data analyses were performed using the statistical software Genstat version 12.2 (VSN International). All statistical results tables are presented in Appendix L.

5.3 Results

5.3.1 Effect of the presence of host and non-host insect on persistence of F418 *gfp* tr3 in soil

5.3.1.1 August 2009 experiment results

The effect of the presence of host and non-host insect on F418 *gfp* tr3 populations in pasteurised and non-sterile soil at 22 % GWC was assessed over 12 weeks (Table 5.1 and Fig 5.2). F418 *gfp* tr3 populations increased in pasteurised soil with host insect and were significantly higher than the INIL at 4, 8 and 12 weeks and there were 348 % of the INIL at 12 weeks. F418 *gfp* tr3 population in pasteurised soil without insects was not significantly different from the INIL at all sampling times. F418 *gfp* tr3 population decreased in

pasteurised soil with the non-host insect and was significantly lower than the INIL at 12 weeks with 42 % of the INIL left. F418 *gfp* tr3 population in pasteurised soil with host insects was significantly higher than with non-host insects and without insects at 4, 8 and 12 weeks. F418 *gfp* tr3 population in pasteurised soil with non-host insect was significantly lower than without an insect at 8 and 12 weeks.

In non-sterile soil, F418 *gfp* tr3 populations in the presence of host insects were not different from the INIL between 0 and 4 weeks followed by a significant increase at 8 and 12 weeks with 277 % of the INIL at 12 weeks. F418 *gfp* tr3 populations in non-sterile soil with non-host insects and without insects were not significantly different from the INIL at all sampling times. F418 *gfp* tr3 populations in non-sterile soil with non-host insects were not different from populations without insects at all sampling times. F418 *gfp* tr3 populations in non-sterile soil with host insects were significantly higher than with non-host insects and without insects at 8 and 12 weeks. F418 *gfp* tr3 populations in pasteurised soil with host insects were significantly higher than in non-sterile soil with host insects between at 4 and 8 weeks but there was no difference at 12 weeks.

Table 5.1 F418 *gfp* tr3 populations recovered (CFU/g DWS and $\log_{10}$ value) in pasteurised and non-sterile soil with host, non-host or without insects at 22 % GWC and held at 15 °C over 12 weeks.

CFU/g DWS **($\log_{10}$)**	Week 0	Week 1	Week 2	Week 4	Week 8	Week 12
Pasteurised soil + host	3.23×10^5 **(5.51)**	4.47×10^5 **(5.65)**	6.15×10^5 **(5.79)**	1.19×10^6 **(6.08)**	2.69×10^6 **(6.43)**	1.12×10^6 **(6.05)**
Pasteurised soil + non-host	3.77×10^5 **(5.58)**	2.23×10^5 **(5.35)**	3.05×10^5 **(5.48)**	2.94×10^5 **(5.47)**	1.72×10^5 **(5.24)**	1.59×10^5 **(5.20)**
Pasteurised soil no insect	3.45×10^5 **(5.54)**	4.05×10^5 **(5.61)**	5.02×10^5 **(5.70)**	4.55×10^5 **(5.66)**	5.25×0^5 **(5.72)**	3.66×0^5 **(5.56)**
Non-sterile soil + host	3.42×10^5 **(5.53)**	2.79×10^5 **(5.45)**	3.08×10^5 **(5.49)**	2.64×10^5 **(5.42)**	8.26×0^5 **(5.92)**	9.48×0^5 **(5.98)**
Non-sterile soil + non-host	3.44×10^5 **(5.54)**	3.60×10^5 **(5.56)**	2.81×10^5 **(5.45)**	2.55×10^5 **(5.41)**	2.25×0^5 **(5.35)**	2.59×0^5 **(5.41)**
Non-sterile soil no insect	3.36×10^5 **(5.53)**	3.40×10^5 **(5.53)**	3.03×10^5 **(5.48)**	2.61×10^5 **(5.42)**	2.50×0^5 **(5.40)**	2.59×10^5 **(5.41)**
LSD ($P < 0.05$)			**0.35**			

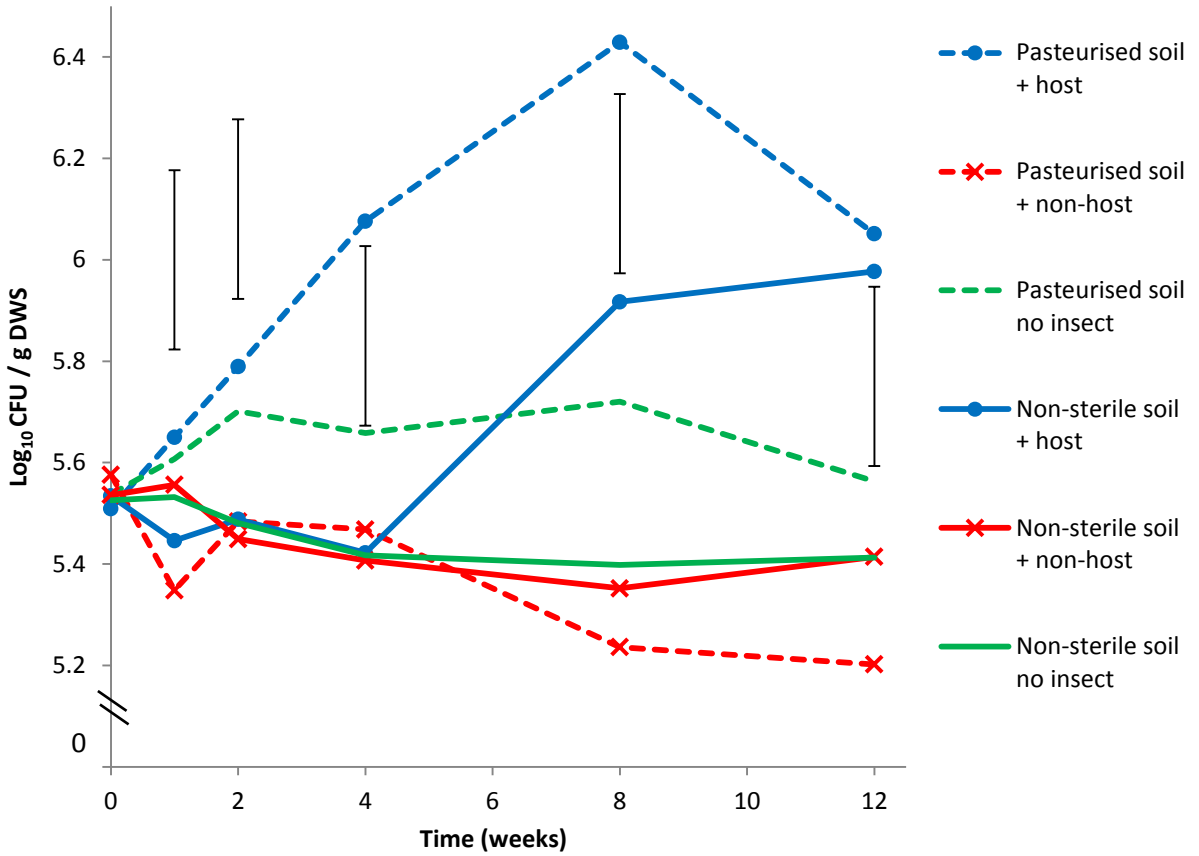

Figure 5.2 F418 *gfp* tr3 populations recovered (log_{10} CFU/g DWS) in pasteurised and non-sterile soil at 22 % GWC and 15 °C in the presence of host, non-host or without insects over 12 weeks in laboratory microcosms. Vertical bar are LSD ($P < 0.05$).

It should be noted that late 3rd instar *C. zealandica* larvae were used in this experiment and the insect went through pupation and were adults before the end of the experiment. Also, the pasteurised and non-sterile soil were at 22 % GWC which is -0.9 bar for pasteurised soil and -2.2 bar for non-sterile soil which means different water availability in the two soils.

5.3.1.2 Soil suspension observation under fluorescence microscopy

From all the soil suspensions observed during the 2009 experiment, very few conidia could be observed directly and the fluorescence was faint. Some clusters of conidia and pieces of mycelium could be observed in soil suspensions from pasteurised soil with host insect at the week 4 sampling (Fig. 5.3).

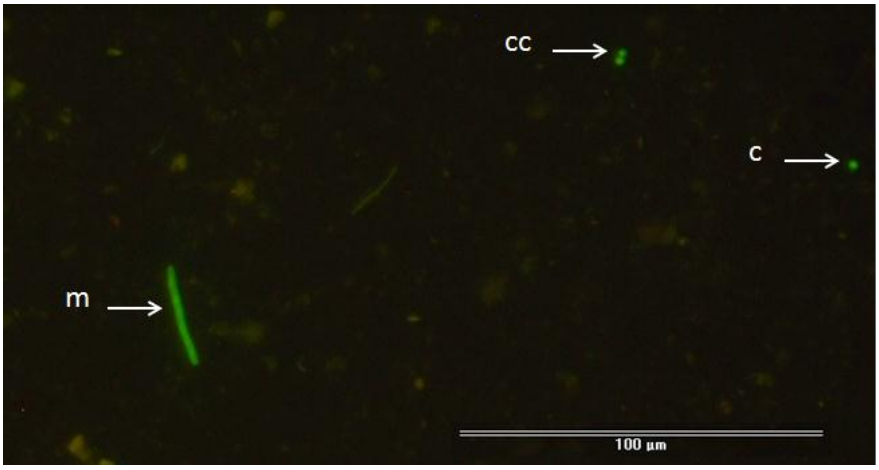

Figure 5.3 Fluorescence microscopy photograph of soil dilution from a container with pasteurised soil in presence of a host insect showing one F418 *gfp* tr3 conidium (c), a cluster of two conidia (cc) and a piece of mycelia (m) during the week 4 sampling of the 2009 experiment.

5.3.1.3 March 2010 experiment results

The effect of the presence of host and non-host insects on F418 *gfp* tr3 populations in pasteurised and non-sterile soil at -2 bar was assessed over 16 weeks (Table 5.2 and Fig 5.4). F418 *gfp* tr3 populations increased in pasteurised soil with host insects and were significantly higher than the INIL between 4 and 16 weeks with 11,722 % of the INIL at 16 weeks. F418 *gfp* tr3 populations in pasteurised soil without insects were significantly higher than the INIL between 4 and 16 weeks with 401 % of the INIL at 16 weeks. However, the populations in soil without insects were significantly lower than with host insects at 8, 12 and 16 weeks. F418 *gfp* tr3 populations in pasteurised soil with non-host insects were not significantly different from the INIL at all sampling times, but were significantly lower than without insects at 8, 12 and 16 weeks.

In non-sterile soil, F418 *gfp* tr3 populations in the presence of host insects were not different from the INIL between 0 and 4 weeks followed by a significant increase at 8, 12 and 16 weeks with 2,831 % of the INIL recovered at 16 weeks. F418 *gfp* tr3 populations in non-sterile soil with non-host insects and without insects were not significantly different from the INIL and from each other at all sampling time. F418 *gfp* tr3 populations in non-sterile soil with host insects were significantly higher than with non-host insects and without insects at 8, 12 and 16 weeks.

F418 *gfp* tr3 populations in pasteurised soil with host insects were significantly higher than in non-sterile soil with host insects between 2 and 16 weeks.

Table 5.2 F418 *gfp* tr3 populations recovered (CFU/g DWS and $\log_{10}$ value) in pasteurised and non-sterile soil at -2 bar and held at 15 °C with host, non-host or without insects over 16 weeks.

CFU/g DWS **($\log_{10}$)**	Week 0	Week 1	Week 2	Week 4	Week 8	Week 12	Week 16
Pasteurised soil + host	1.55×10^5 **(5.19)**	1.73×10^5 **(5.24)**	3.77×10^5 **(5.58)**	6.65×10^5 **(5.82)**	2.52×10^6 **(6.40)**	6.73×10^7 **(7.83)**	1.81×10^7 **(7.26)**
Pasteurised soil + non-host	1.47×10^5 **(5.17)**	1.14×10^5 **(5.06)**	3.25×10^5 **(5.51)**	3.13×10^5 **(5.50)**	1.48×10^5 **(5.17)**	2.26×10^5 **(5.35)**	2.13×10^5 **(5.33)**
Pasteurised soil no insect	1.46×10^5 **(5.17)**	3.33×10^5 **(5.52)**	3.48×10^5 **(5.54)**	5.68×10^5 **(5.75)**	6.62×10^5 **(5.82)**	7.48×10^5 **(5.87)**	5.86×10^5 **(5.77)**
Non-sterile soil + host	1.46×10^5 **(5.17)**	1.56×10^5 **(5.19)**	1.20×10^5 **(5.08)**	1.67×10^5 **(5.22)**	5.14×10^5 **(5.71)**	8.69×10^5 **(5.94)**	4.14×10^6 **(6.62)**
Non-sterile soil + non-host	1.45×10^5 **(5.16)**	1.54×10^5 **(5.19)**	1.29×10^5 **(5.11)**	1.00×10^5 **(5.00)**	9.51×10^4 **(4.98)**	9.14×10^4 **(4.96)**	9.46×10^4 **(4.98)**
Non-sterile soil no insect	1.30×10^5 **(5.11)**	1.19×10^5 **(5.08)**	1.24×10^5 **(5.09)**	1.20×10^5 **(5.08)**	9.31×10^4 **(4.97)**	1.22×10^5 **(5.09)**	1.13×10^5 **(5.05)**
LSD ($P < 0.05$)				**0.39**			

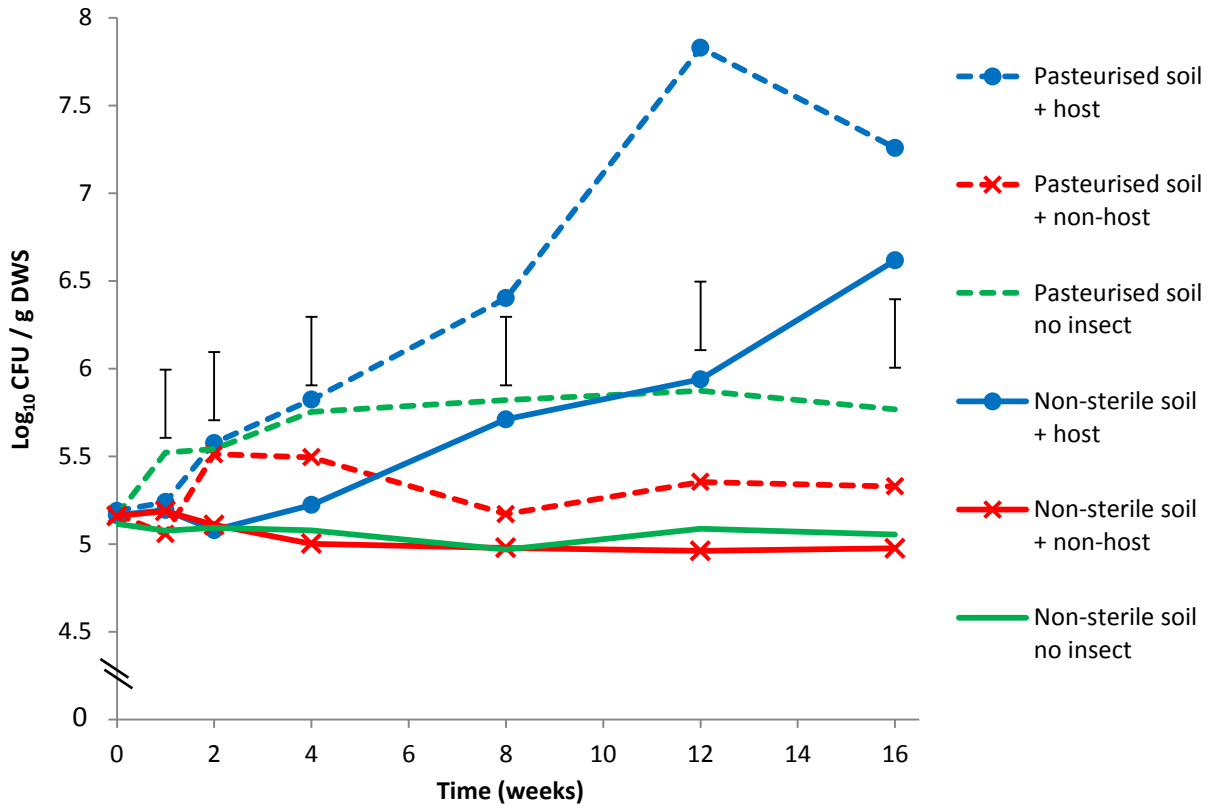

Figure 5.4 F418 *gfp* tr3 populations recovered (log_{10} CFU/g DWS) in pasteurised and non-sterile soil at -2 bar and held at 15 °C in the presence of host, non-host or without insects in laboratory microcosms. Vertical bars are LSD (P < 0.05).

5.3.1.4 Conidiation on dead *T. molitor* larvae

At 2 weeks, a larva was found dead in a container of pasteurised soil but it did not support conidiation (Fig 5.5) and was not kept to check whether F418 *gfp* tr3 conidia were produced from it. However the larvae presented signs of internal mycelium infection and was therefore assumed to have died from F418 *gfp* tr3 infection. All the larvae in the other containers (pasteurised and non-sterile soil) were still alive at 2 weeks.

All *T. molitor* larvae were found dead in both pasteurised and non-sterile soil containers at 4 weeks. None showed sign of conidiation but it was assumed that they died from F418 *gfp* tr3, although it cannot be confirmed as there was no conidiation.

At 8 weeks, all larvae from pasteurised and non-sterile soil were dead. Larvae from pasteurised soil were all supporting conidiation (light to heavy conidiation). One larva from the non-sterile soil was dead but from *M. anisopliae* (as the larva was

covered in green conidia) and the three other larvae showed conidiation from F418 *gfp* tr3 ranging from light to medium conidiation.

At 12 weeks, all larvae from both soil containers were dead and larvae from pasteurised soil were all supporting heavy conidiation. One larva from non-sterile soil was dead but from *M. anisopliae* and the three other larvae showed conidiation from F418 *gfp* tr3 ranging from light to medium conidiation. At 16 weeks, all larvae from both soils were dead and the fungus in most larvae from pasteurised soil was sporulating heavily while larvae from non-sterile soil had mainly a medium level of conidiation.

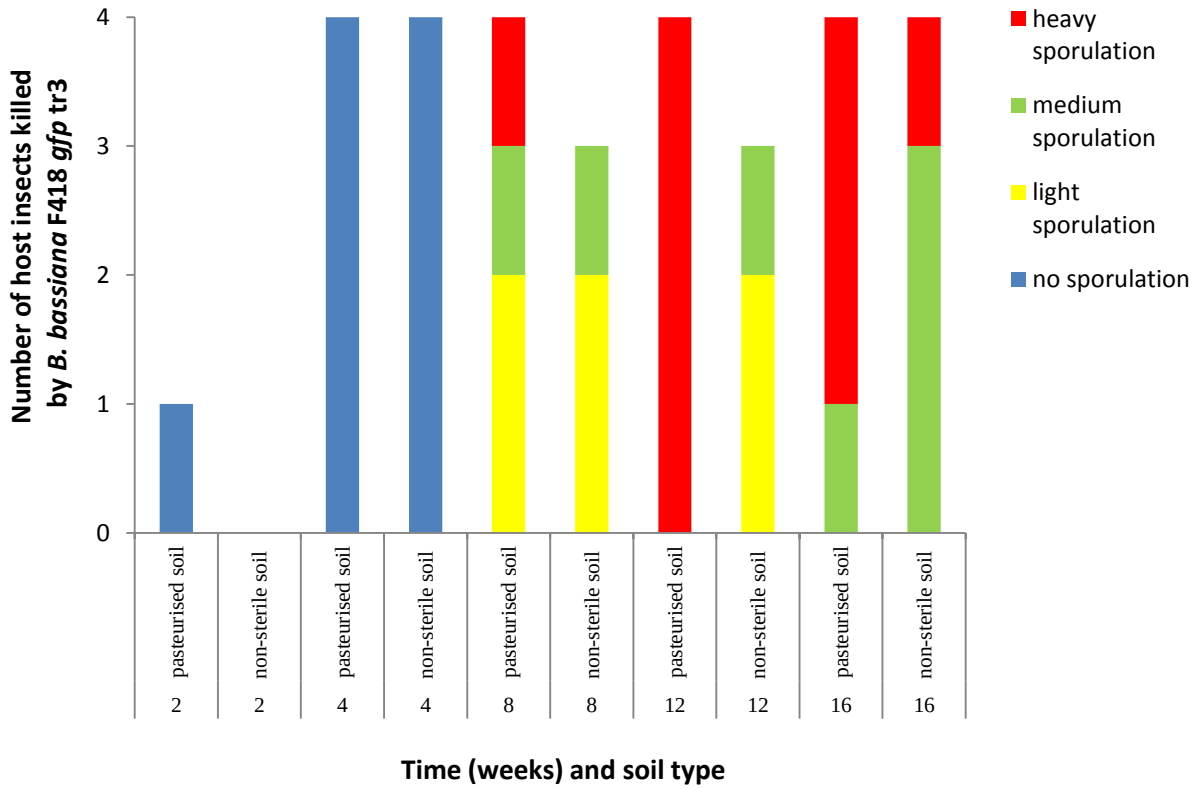

Figure 5.5 Number of host insects *T. molitor* killed by F418 *gfp* tr3 in pasteurised and non-sterile soil and their degree of conidiation at different sampling times (n=40).

5.3.1.5 Observation of germination on non-host *C. zealandica* larvae cuticle

The observation of the non-host insect (*C. zealandica* larvae) cuticles sampled from soil did not show any attached and/or germinated conidia.

5.3.2 Fungal and bacterial communities in soil with host, non-host and no insects

5.3.2.1 Bacterial communities in soil

Bacterial banding patterns on SSCP gels from pasteurised soil were different between the beginning and the end of the experiment (Table 5.3 and Fig. 5.6). A few bands such as 1, 2 and 3 were dominant at the beginning of the experiment. At the end of the experiment, bacterial banding patterns in pasteurised soil with host insects were similar to banding patterns without insects, whereas banding patterns with non-host insects were different with the presence of unique bands such as bands 4 and 8. There were common bands in pasteurised soil with hosts and without insects such as bands 5 and 6.

There was no difference in bacterial banding patterns in non-sterile soil with different insect treatments and time. The diversity of bacterial bands was much greater in non-sterile soil than in pasteurised soil.

Table 5.3 Sequences matching of bacterial and fungal DNA extracted from SSCP gel

Bands number	Sequence matches	Bands number	Sequence matches
1, 2, 3	*Lactobacillus* spp. Beijerinck (Lactobacillales: Lactobacillaceae)	63	*Preussia* sp. Fuckel (Ascomycota: Pleosporales)
5	*Micromonospora* sp.	51, 57	*Gelasinospora tetrasperma* Dowding (Ascomycota: Sordariales)
6	*Trichosporon porosum* (Stautz) W.J. Middelhoven, G. Scorzetti & J.W. Fell (Basidiomycota: Tremellales)	64, 65, 66	*Trichosporon* spp. Behrend (Basidiomycota: Tremellales)
4, 8, 7, 10, 11, 54, 55, 56	Sequence results ambiguous		

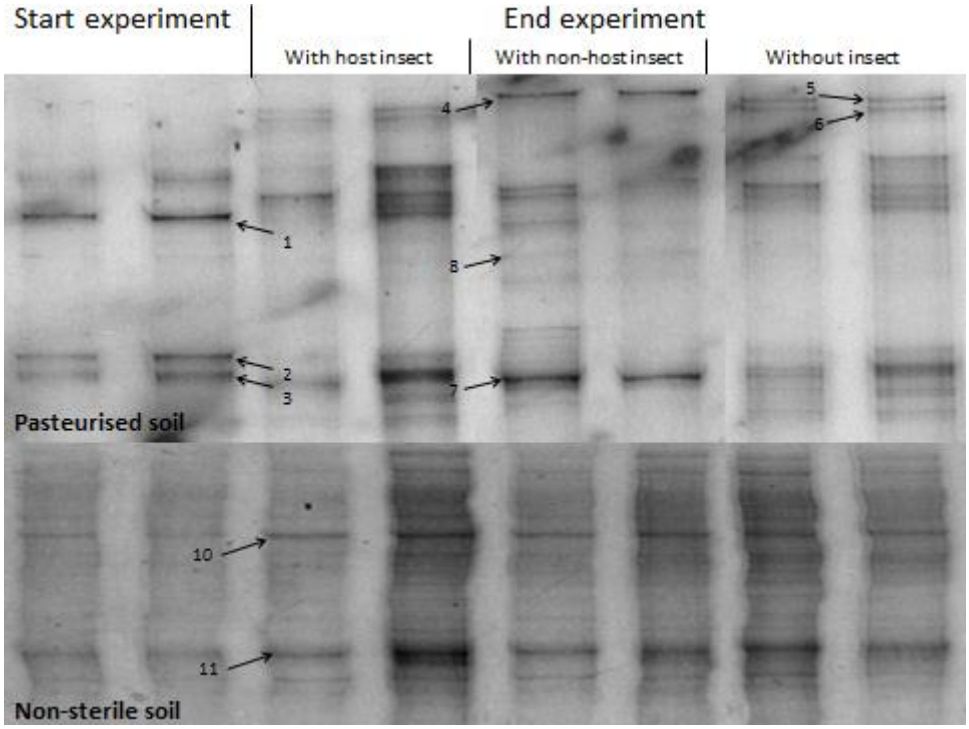

Figure 5.6 SSCP banding patterns of bacterial communities in pasteurised and non-sterile soils at the beginning and the end of the experiment with host, non-host and without insects. Arrows indicate sequenced bands.

5.3.2.2 Fungal communities in soil

Fungal bandings patterns in pasteurised soil showed few differences between the beginning of the experiment and the end (Table 5.3 and Fig. 5.7). Bands 51 and 57 were present at the beginning of the experiment. Bands 54, 55 and 56 were common in most treatments and time points but the sequences obtained were ambiguous. There were some differences in fungal banding patterns between the different insect treatments at the end of the experiment. Bands 63, 64, 65 and 66 were only present in pasteurised soil with non-host insects, although not in both replicates. Bands corresponding to F418 *gfp* tr3 (i.e. 53, 58, 59, 60, 61 and 62) were present in all treatments at the end of the experiment but their intensity differed. Only bands matching F418 *gfp* tr3 could be seen in pasteurised soil with host insects at the end of the experiment.

Fungal banding patterns in non-sterile soil showed that many bands were common to all treatments and times. Bands corresponding to F418 *gfp* tr3 were clearly present in one of the replicate from non-sterile soil with host insects. The diversity of fungal bands was greater in non-sterile soil than in pasteurised soil.

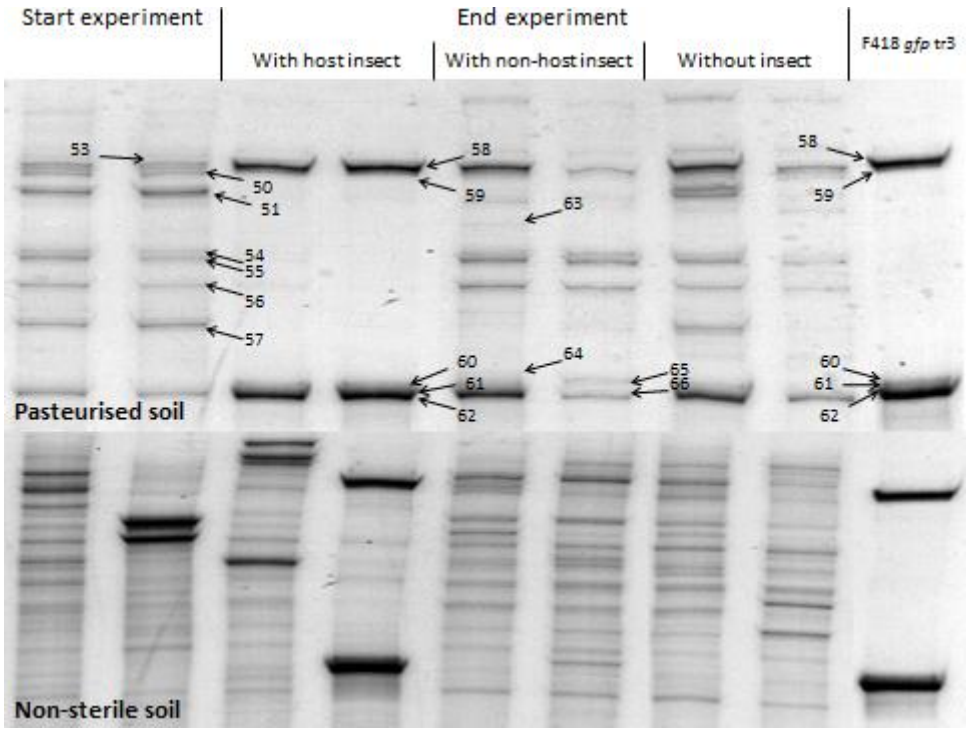

Figure 5.7 SSCP banding patterns of fungal communities in pasteurised and non-sterile soils at the beginning and the end of the experiment with host, non-host and without insects. Arrows indicate sequenced bands.

5.3.3 Germination of F418 *gfp* tr3 on *C. zealandica* larvae cuticle

5.3.3.1 F418 *gfp* tr3 germination on *C. zealandica* larvae ghost cuticle

F418 *gfp* tr3 conidia attachment and germination was observed on *C. zealandica* larvae ghost cuticle after 1 day of incubation at room temperature (20 °C ± 2) (Fig. 5.8). F418 *gfp* tr3 conidia could be observed using fluorescence microscopy but the fluorescence was faint (Fig. 5.9b). All ghost cuticles observed had germinated conidia on them.

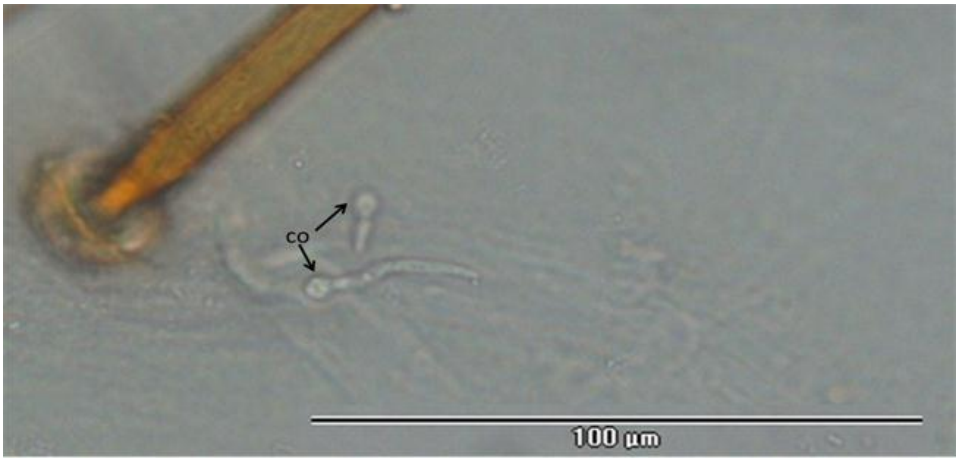

Figure 5.8 Light microscopy photograph of F418 *gfp* tr3 conidia (co) germinating beside a root hair (rh) on washed ghost cuticle of *C. zealandica* larvae after 1 day of incubation at room temperature (20 °C ± 2).

F418 *gfp* tr3 appressorium and penetration peg formation were observed after 2 d of incubation on *C. zealandica* larvae ghost cuticle incubated at room temperature (20 °C ± 2) (Fig 5.9 and 5.10).

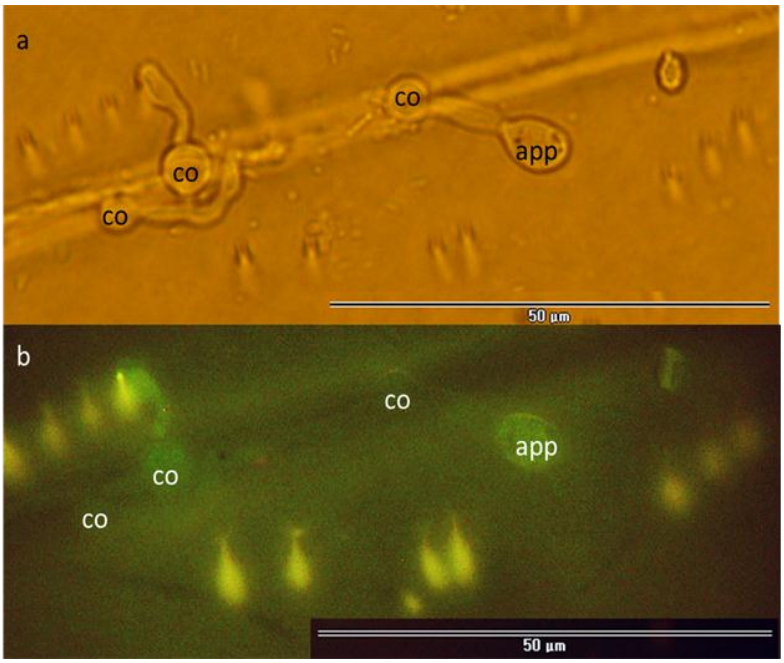

Figure 5.9 (a) Light microscopy photograph and (b) fluorescence microscopy photograph of F418 *gfp* tr3 (co) conidia germinating on washed ghost cuticle of *C. zealandica* larvae and conidium producing (ap) an appressorium after 2 d of incubation at room temperature (20 °C ± 2).

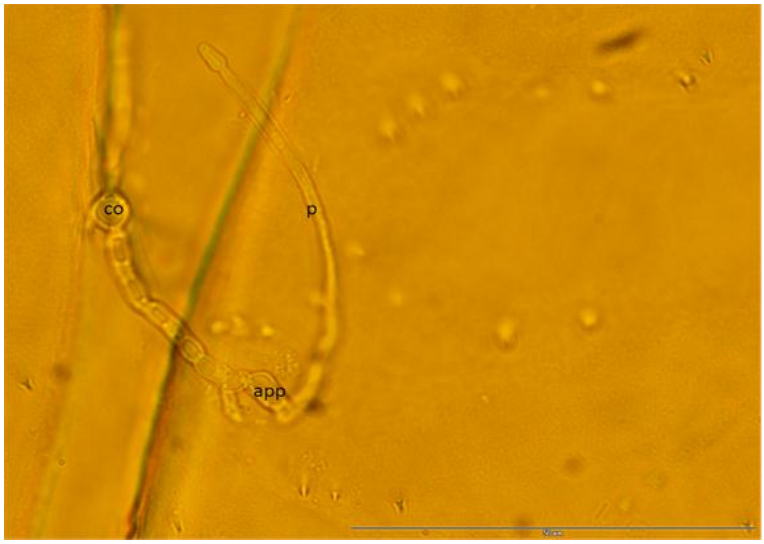

Figure 5.10 Light microscopy photograph of F418 *gfp* tr3 (co) conidium germinating on washed ghost cuticle of *C. zealandica* larvae and conidium producing (app) an appressorium and (p) penetration peg after 2 d of incubation at room temperature (20 °C ± 2).

5.3.3.2 F418 *gfp* tr3 germination on live *C. zealandica* larva cuticle

F418 *gfp* tr3 conidial attachment and germination was observed on *C. zealandica* larvae cuticle inoculated alive by the immersion, drop and the filter inoculation method after 1 and 2 d of incubation at room temperature (20 °C ± 2) (Fig. 5.11 to 5.13). All cuticles observed showed attached and germinated F418 *gfp* tr3 conidia with the three different inoculation methods. F418 *gfp* tr3 conidia could be observed using fluorescence microscopy but the fluorescence was faint (Fig. 5.12b).

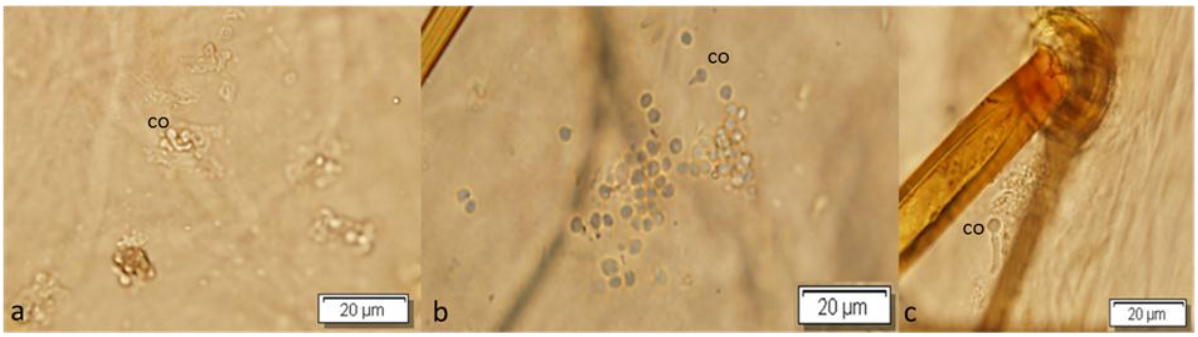

Figure 5.11 Light microscopy photographs of F418 *gfp* tr3 conidia (co) germinating on cuticle of live *C. zealandica* larvae inoculated by (a) immersion, (b) drop method and (c) filter method after 1 day of incubation at room temperature (20 °C ± 2).

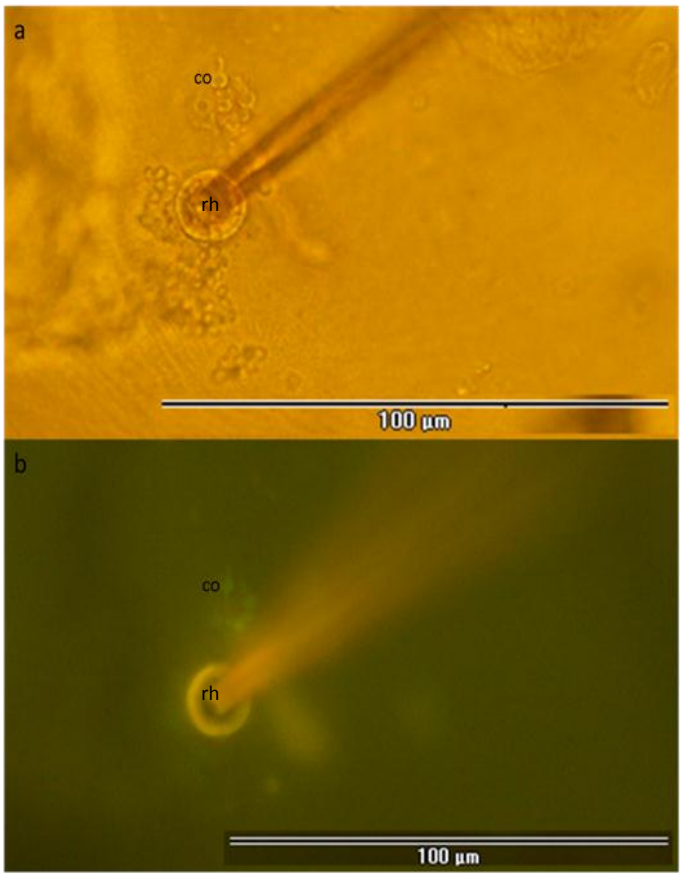

Figure 5.12 (a) Light and (b) fluorescence microscopy photographs of F418 *gfp* tr3 conidia (co) germinating on cuticle of live *C. zealandica* larvae near a root hair (rh) inoculated by immersion method after 1 day of incubation at room temperature (20 °C ± 2).

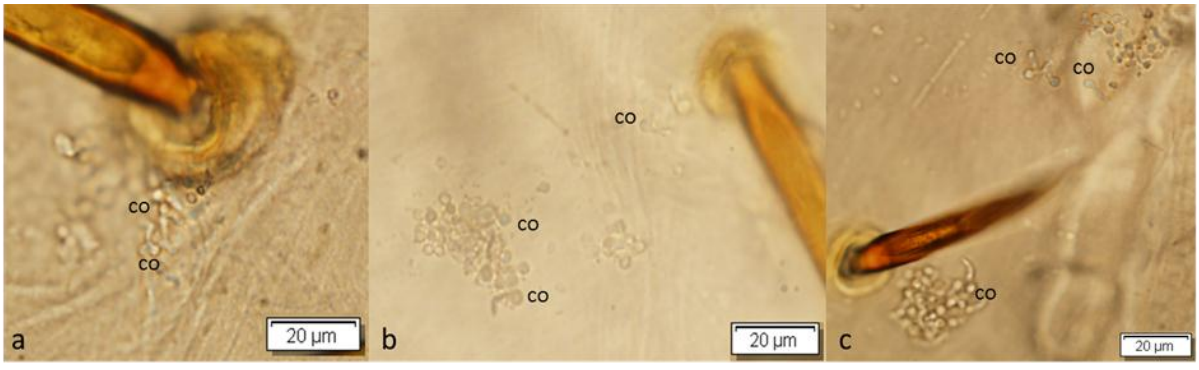

Figure 5.13 Light microscopy photographs of F418 *gfp* tr3 conidia (co) germinating on cuticle of live *C. zealandica* larvae inoculated by (a) immersion, (b) drop method and (c) filter method after 2 d of incubation at room temperature (20 °C ± 2).

F418 *gfp* tr3 conidial attachment and germination was observed on all *C. zealandica* larvae cuticle infected alive by immersion and drop method after 2 d of incubation at 15 °C (Fig. 5.14).

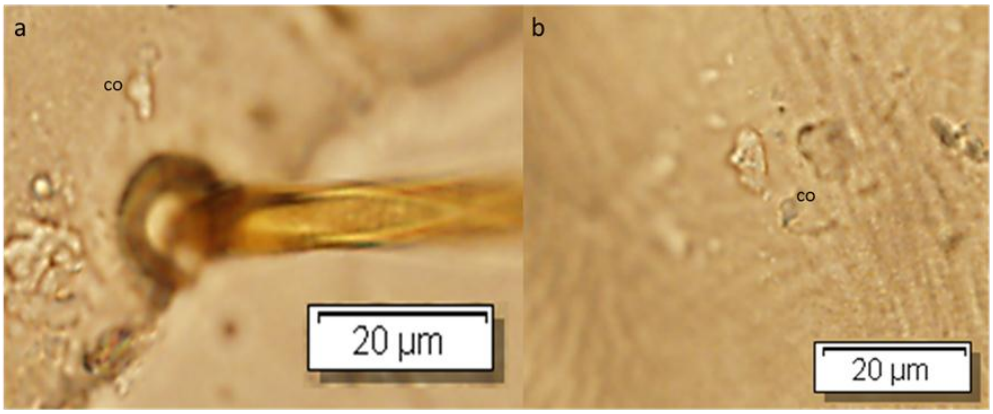

Figure 5.14 Light microscopy photographs of F418 *gfp* tr3 conidia (co) germinating on cuticle of live *C. zealandica* larvae inoculated by (a) immersion and (b) drop method after 2 d in a 15 °C incubator in the dark.

F418 *gfp* tr3 appressorium formation was observed on *C. zealandica* larvae cuticle inoculated live by immersion after 3 d of incubation at room temperature (20 °C ± 2) (Fig. 5.15).

Figure 5.15 Light microscopy photograph of F418 *gfp* tr3 conidium (co) germinating on cuticle of live *C. zealandica* larvae and producing (ap) an appressorium after inoculation by immersion after 3 d of incubation at 20 °C ± 2.

5.3.4 Recovery of F418 *gfp* tr3 CFU after conidia ingestion by *C. zealandica* larvae

5.3.4.1 Conidia recovery in faeces

A total of 91 *C. zealandica* larvae were force fed F418 *gfp* tr3 conidia suspension in four different experiments but F418 *gfp* tr3 CFU recovery in faeces was made from 70 larvae as some larvae died or did not produce any faeces during the experiment and could not be considered for the data analysis. CFU from F418 *gfp* tr3 recovered after ingestion by *C. zealandica* larvae ranged from 3.4 to 100 % recovery (Fig. 5.16). There was no null total recovery of F418 *gfp* tr3 CFU from larvae faeces. For 21 % (15 out of 70) of the larvae, 91 to 100 % of the ingested F418 *gfp* tr3 CFU were recovered. For 51 % of the larvae, more than half of the ingested F418 *gfp* tr3 conidia were recovered. Most of the CFU recovered were from faeces collected during the first few days after ingestion (under 7 days), therefore CFU recovered could not be from a multiplication of F418 *gfp* tr3 inside *C. zealandica* larvae guts. Most larvae were covered in their own faeces during the daily faeces sampling.

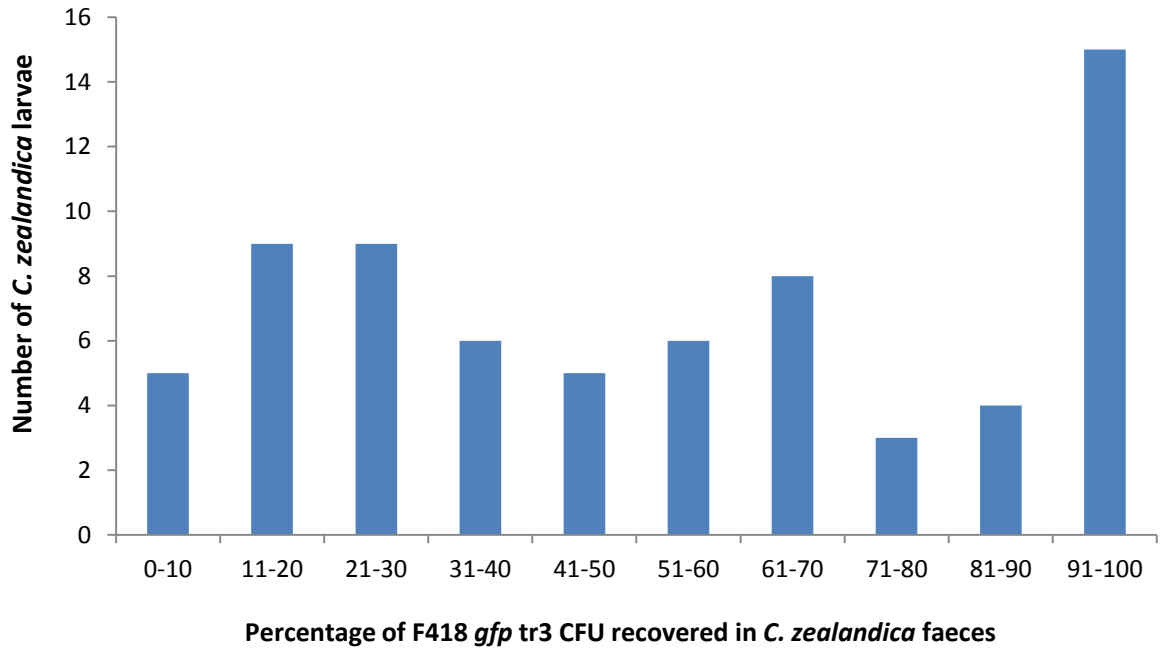

Figure 5.16 Percentage of F418 *gfp* tr3 CFU recovery in faeces after conidia ingestion by *C. zealandica* larvae and the number of larvae in each recovery range (n=70).

5.3.4.2 Cuticle observation

No conidia could be seen on any of the cuticles from force-fed larvae observed under fluorescence microscopy.

5.4 Discussion

5.4.1 Germination of F418 *gfp* tr3 conidia on *C. zealandica* cuticle

F418 *gfp* tr3 conidia could attach and germinate on non-host *C. zealandica* ghost cuticle within one day and produce appressorium and penetration peg within 2 d. F418 (and its transformants) is not pathogenic to *C. zealandica*. Boucias *et al*. (1988) found that conidia from entomopathogenic fungi *Nomuraea rileyi* (Farl.) Samson (Ascomycota: Hypocreales), *B. bassiana* and *M. anisopliae* could attach to the ghost cuticle of velvetbean caterpillar larvae *A. gemmatalis* which is a host for *N. rileyi* but a non-host for *B. bassiana* and *M. anisopliae*. Boucias *et al*. found that all three fungi could attach to velvetbean caterpillar larvae ghost cuticles irrespectively of being a host or non-host of the fungus. Their study showed that fungal conidia were strongly attached to ghost cuticles by hydrophobic interactions and that the conidial attachment is passive and not due to cues from the host arthropod cuticle.

In this study, the germination experiment was then done on live *C. zealandica* larvae as there is no immune system in ghost cuticles which could affect the interaction between the fungal conidia and the non-host cuticle (Gillespie *et al*., 2000; Vilcinskas & Götz, 1999). The germination assay on live *C. zealandica* showed that F418 *gfp* tr3 conidia could still attach and germinate on live non-host cuticle within 1 day at room temperature (20 °C ± 2) and within 2 d at 15 °C, and appressorium formation could be observed at room temperature after 3 d of incubation. Studies by Sitch & Jackson (1997) showed that *L. lecanii* (formerly *Verticillium lecanii*) conidia could germinate at the same rate and produce germ-tubes on the cuticle of host aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae) and the non-host adult *B. obtusum* 30 h post-infection, although spore retention was greater on host arthropods compared to non-host arthropods. Their findings indicate that resistance mechanisms in non-host arthropods must intervene after fungal germination. Similarly, studies from Hajek & Eastburn (2003) showed that *E. maimaiga* conidia could germinate on the surface of

non-host *Chaetaglaea sericea* Franclemont (Lepidoptera: Noctuidae) 12 h post infection, although germination rate was lower than on host cuticle of *Lymantra dispar* L. (Lepidoptera: Erebidae). Ment *et al.* (2012) showed that *M. anisopliae* could germinate on non-host cuticle of ticks (Acari: Ixodidae) but could not penetrate the cuticle and died after a few days.

The use of different techniques for the infection of the live non-host larvae (immersion, drop or inoculated filter) in the present study did not seem to alter the interaction between F418 *gfp* tr3 conidia and the non-host *C. zealandica* cuticle. Sitch & Jackson (1997) used a Potter tower to spray *L. lecanii* on host and non-host arthropods. Hajek & Eastburn (2003) used sporulating cadavers of *L. dispar* to shower *E. maimaiga* onto host and non-host arthropods. The technique used to infect non-host arthropods does not seem to be very important as long as there is enough contact time between the conidia and the cuticle. Boucias *et al.* (1988) found that a strong binding between conidia and non-host ghost cuticle was obtained immediately upon contact and conidia in contact with the cuticle for less than 5 min could not be removed by boiling in 1 % sodium dodecyl sulphate.

The current study showed that F418 *gfp* tr3 conidia can attach and germinate on non-host cuticle of live *C. zealandica* larvae. F418 *gfp* tr3 is not pathogenic to *C. zealandica* therefore conidia on the non-host cuticle probably die leading to a loss of viable conidia (Ment *et al*., 2012). *Costelytra zealandica* is an important pastoral pest in New Zealand (Jackson *et al*., 1993) as well as the weevil *S. lepidus*. The *C. zealandica* larval density used in the present experiment was equivalent to nearly 300 larvae/m^2 which is over the threshold density for damage of 200 larvae/m^2 and is a medium density considering the maximum density of approximately 1,500 larvae/m^2. The possibility that F418 *gfp* tr3 can germinate on *C. zealandica* cuticle means that there could be a potential issue of conidia loss after application of F418 for the control of *S. lepidus* in pasture considering a high density of *C. zealandica* present in New Zealand pastures.

5.4.2 Recovery of F418 *gfp* tr3 conidia ingested by *C. zealandica* larvae

The ingestion experiment showed that F418 *gfp* tr3 conidia could be recovered from all *C. zealandica* larvae after ingestion and that the majority of conidia (91 to 100 % CFU recovered) were recovered from 21 % of the larvae. Dromph (2001) showed that 95.6, 100 and 98.8 % of the faecal pellets recovered respectively from the non-host collembolans *P. minuta*, *F. fimetaria* and *Hypogastrura assimilis* Krausbauer (Collembola: Hypogastruridae) gave rise to *B. bassiana* (KVL 98-22) colonies. In the same study, Dromph

also found that *B. bassiana* conidia recovered from faeces had the same germination rate as non-ingested conidia. However, collembolans were observed freely feeding on the fungal spores and the number of conidia ingested was unknown; therefore the rate of *B. bassiana* conidia recovery could not be measured. There is no published work in which a known number of fungal conidia were force-fed to an arthropod to determine the percentage of viable conidia after digestion, as was done in the current study.

In the current study, non-host faeces were collected daily up to 11 d in the three different experiments and F418 *gfp* tr3 colonies grew from the faeces collected between 1 and 11 d, although not every insect's faeces collected gave rise to colonies at all sampling times. There is no published paper about conidia viability and germination after passage through arthropod guts in which authors sampled the faeces for up to 11 d. Most works have only studied conidia recovery from few hours to 5 d post-feeding. Schabel (1976) found that conidia of *M. anisopliae* fed on contaminated pine twigs could be found in sections of the guts of host insect *H. pales* beetles up to 120 h (5 d) post ingestion. Also in the same study, conidia recovered from faeces and guts up to 86 h (3.5 d) post digestion were still viable. This study showed that ingestion of F418 *gfp* tr3 by non-host *C. zealandica* larvae is likely to have no detrimental effect on the viability of F418 *gfp* tr3 propagules.

The current study indicates that F418 *gfp* tr3 conidia can be retained in the non-host *C. zealandica* larvae guts for up to 11 d and still be viable. Faeces were not collected after 11 d and the guts content was not recovered and plated, therefore it is possible that more viable conidia were still present in the digestive system of *C. zealandica* larvae after the last faeces collection, which could partially explain the lack of full recovery of viable conidia from all insects.

The faeces were sampled daily and the larvae were usually covered in their own faeces. As discussed in the previous section, F418 *gfp* tr3 conidia can attach and germinate on the cuticle of non-host *C. zealandica* larvae. The strong binding of conidia to the larvae cuticle is probably the main explanation for the low conidial recovery for some insects as conidia could not be washed off the cuticle once attached and could not be plated. However, *C. zealandica* larvae were kept after the ingestion experiment for cuticle observation and no conidia could be observed on the cuticle. Although, only approximately 2,400 conidia were force fed and even if all of them were attached to the cuticle, it would still be a very low number of conidia for the size of the cuticle surface. In comparison, in the germination experiment, the larvae cuticles were in contact with 1×10^6 conidia per larvae in the drop method and the observation of attached and germinated conidia could be done. Also, in the ingestion experiment, the larvae were assessed at the end of the experiment (9 to 11 d) and any

conidia that may have attached to the cuticle in the early stages of the experiment could have been degraded by then (Ment *et al*., 2012) and difficult to observe. Even though the observation of attached conidia could not be made to confirm the hypothesis, it is likely that conidia from the faeces attached to the cuticle leading to a lower recovery rate for some insects.

The capacity of F418 *gfp* tr3 conidia to pass through *C. zealandica* larvae guts while remaining viable indicates the potential for dispersal of F418 via *C. zealandica* larvae in the pasture. *Costelytra zealandica* larvae and *S. lepidus* larvae are both present in pasture soil from late autumn to early spring and *C. zealandica* larvae eat soil particles while feeding on plant roots (Allison, 1969), therefore, it is likely that *C. zealandica* larvae would ingest F418 in treated pasture and would therefore disperse it in their surrounding environment, helping with the dispersal F418 for the biocontrol of clover root weevil.

5.4.3 Impact of the presence of host or non-host insect on microbial communities in soil

Fungal and bacterial communities were affected by the soil treatment (pasteurised and non-sterile soil) and by the presence of insects. Bacterial communities in pasteurised soil changed from the beginning to the end of the experiment and the change was different with non-host insects compared to host or without insects. As discussed in Chapter 4, the microbial niche left empty after the pasteurisation process were recolonised with new microbes over time. It seems that the microbial recolonisation process was different in soil with non-host insects compared to soil with host or without insects. There is no literature about microbial communities in soil with different type of arthropods. Larvae of *T. molitor* were laboratory reared and were probably carrying fewer microbes than *C. zealandica* larvae which were field collected with guts full of non-sterile soil and thus a large microbial population (Zhang & Jackson, 2008). Therefore, this could explain why bacterial communities in pasteurised soil with host insects were similar to without insects, whereas bacterial communities in pasteurised soil with non-host insects were clearly different; probably due to the bacteria present on and inside the *C. zealandica* larvae. Bacterial communities in non-sterile soil were similar in all treatments and times and as discussed in Chapter 4, the microbial niches were probably full in non-sterile soil and any new microbes brought by host or non-host insects would have to compete with the already present fauna

which was adapted to the soil environment, therefore the stable bacterial communities in all non-sterile soil.

Fungal communities in pasteurised soil were quite similar between the start and the end of the experiment in soil with non-host and without insects, excepted for few bands which seemed to be present in soil with non-hosts only. The main difference in fungal communities in pasteurised soil was the presence of F418 *gfp* tr3 bands which were clearly stronger in soil with host insects compared to non-host and without insects. The presence of strong bands matching F418 *gfp* tr3 correlated to F418 *gfp* tr3 CFU recovery in pasteurised soil with host insects which was most likely due to the production of F418 *gfp* tr3 conidia on the dead host insects. There were no other fungal bands present in pasteurised soil with host insects. This probably does not mean that there were no other fungi present but that the quantity of F418 *gfp* tr3 was so much higher compared to any other fungi that it outcompeted the DNA from other species during PCR and effectively “hid” the other fungi present.

Fungal communities in non-sterile soil were similar in soil with non-host and without insects. There is no obvious explanation of the fact there were stronger fungal bands in non-sterile soil at the start of the experiment and in the first replicate in soil with host insects. However, there were stronger bands in the second replicate in soil with host insects which correspond to *B. bassiana* and this is most likely due to F418 *gfp* tr3 conidiation on dead *T. molitor* larvae. The difference between the two replicates in non-sterile soil with host insects might be due to the sampling of the soil for the fungal community analysis and the degree of conidiation of the dead larvae. Unlike in the study of F418 *gfp* tr3 persistence with host insects, the dead *T. molitor* larvae were removed from the container before the soil sampling for the fungal community analysis, therefore removing a source of conidia from the container. It is possible that not many conidia fell from the dead insects onto the soil in the first replicate therefore the absence of strong bands for *B. bassiana*.

Bacterial and fungal communities were different in pasteurised and non-sterile soil. As discussed in Chapter 4, bacterial and fungal communities banding patterns in non-sterile soil were higher in numbers than banding patterns from pasteurised soil due to the pasteurisation process which killed most microbes in pasteurised soil and lowered the diversity of them.

5.4.4 Impact of non-host *C. zealandica* on persistence of F418 *gfp* tr3 in soil

The current study is the first to show the persistence of a *B. bassiana* strain in soil in presence of non-host insect and the interaction with non-host insects which could explain the persistence observed.

The results obtained on the persistence of F418 *gfp* tr3 in pasteurised soil with non-host insects in this chapter were similar to those obtained with F418 in Chapter 3. In all three experiments, F418 *gfp* tr3 and F418 CFU recovery in pasteurised soil with non-host *C. zealandica* larvae was significantly lower than without insects. The results presented in this chapter showed that F418 *gfp* tr3 conidia can attach and germinate on non-host *C. zealandica* larvae cuticle, and as *C. zealandica* is not a host for F418 *gfp* tr3, the conidia which attach and germinate on its cuticle would eventually be lost (Ment *et al*., 2012). It is therefore probable that the lower CFU recovery observed in pasteurised soil in presence of non-host insects compared to without insects is due to the non-specific attachment and germination of F418 *gfp* tr3 conidia to cuticle of non-host *C. zealandica* larvae leading to a loss of F418 *gfp* tr3 conidia. The observation of *C. zealandica* larvae cuticle after soil sampling did not show attached or germinated conidia on its surface unlike the *in vitro* experiment. Although, the number of conidia in contact with the *C. zealandica* larvae cuticle at a time in soil is certainly much lower than the quantity used for the germination on cuticle experiments and the number of attached conidia might be insufficient to be observed easily. Also, germinated F418 *gfp* tr3 conidia may eventually die and be degraded on the non-host arthropod cuticle and this process could happen within few days (Ment *et al*., 2012), therefore it might have been difficult to observe germinated conidia on *C. zealandica* larvae cuticle sampled in soil.

The difference in bacterial and fungal communities in pasteurised soil with non-host insects could be responsible for the lower F418 *gfp* tr3 CFU recoveries compared to without insects. Microbial communities (mostly bacterial) were different with non-host insects, which may be due to the presence of microbes in the gut and on the surface of field collected *C. zealandica* larvae. As discussed in Chapter 4, the different bacteria present in pasteurised soil with non-host insects might be antagonistic to F418 *gfp* tr3. Therefore, the presence of different types of bacteria (probably antagonistic) in pasteurised soil with non-host insects could explain the lower CFU recoveries compared to without insects.

Costelytra zealandica larvae used in the 2009 experiment were late 3rd instar which pupated and became adults during the course of the experiment (around 8 and 12 weeks). Unlike 3rd

instar larvae which are eating and moving through the soil, *C. zealandica* pupae are less active in the soil and, once emerged, *C. zealandica* beetles do not move through the soil nor eat soil. Even though there was a change in the non-host stage and behaviour during the experiment, the results found in the 2009 experiment were similar to the results obtained in 2010, possibly because the pupation occurred at a late stage of the experiment.

F418 *gfp* tr3 CFU recovery in pasteurised soil with non-host insects was lower than the INIL in the 2009 experiment, while it was not different from the INIL in the 2010 experiment. There was a difference in water availability in pasteurised soil between the two experiments with pasteurised soil in the 2009 experiment being at 22 % GWC which is equivalent to -0.85 bar while the pasteurised soil in the 2010 experiment was at -2 bar. Therefore the pasteurised soil in 2009 had more water available than the pasteurised soil in the 2010 experiment and as shown in Chapter 4, F418 *gfp* tr3 populations were higher in soils with lower moisture level. This difference in water availability could explain the difference in F418 CFU recovery in pasteurised soil with non-host insects observed in both experiments.

The study of the persistence of F418 *gfp* tr3 in non-sterile soil in the presence of non-host or without insects in 2009 and 2010 gave similar results. In both experiments, there was no significant difference in persistence of F418 *gfp* tr3 in non-sterile soil with non-host and without insects. The findings obtained in non-sterile soil with non-host insects were different from the results obtained in pasteurised soil. Attachment and germination of F418 *gfp* tr3 conidia on *C. zealandica* larvae cuticle or presence of antagonistic microbes could explain the lower F418 *gfp* tr3 CFU recovery in pasteurised soil with non-host insects compared to F418 *gfp* tr3 CFU recovery in pasteurised soil without insects. As there is no difference in CFU recovery in non-sterile soil with non-host or without insects, germination of F418 *gfp* tr3 conidia and subsequent loss on *C. zealandica* larvae cuticle might not be happening in non-sterile soil. Also there was no obvious difference in microbial populations in non-sterile with non-host insects compared to soil without insects. Natural soils are known to be a limiting environment for fungi to germinate and grow due to fungistasis (Lockwood, 1964). Walstad *et al.* (1970) found that *B. bassiana* and *M. anisopliae* conidia did not germinate in unsterilized soil, probably due to inhibition by other microorganisms', but conidia could germinate once the soil was sterilised. They also found that *B. bassiana* and *M. anisopliae* conidia could germinate on unsterile body wall of the host weevil *H. pales*.

Fungistasis in non-sterile soil is likely to prevent the germination of F418 *gfp* tr3 conidia on the cuticle of *C. zealandica* larvae unlike in pasteurised soil, therefore, there could be no loss of conidia via germination on non-host insect. Also, non-sterile soil would be a competitive environment for any antagonist bacteria potentially brought by the non-host and therefore

any antagonistic bacteria could not develop as well in non-sterile soil as in pasteurised soil where ecological niches are available. Fungistasis is likely to explain the F418 *gfp* tr3 CFU recovery in non-sterile soil in presence of non-host insects and without insects.

5.4.5 Impact of host insect *T. molitor* on persistence of F418 *gfp* tr3 in soil

The study of the persistence of F418 *gfp* tr3 in non-sterile soil in the presence of host insects in 2009 and 2010 gave similar results. In both experiments, F418 *gfp* tr3 CFU recovery increased as the host died and supported conidia production.

The study of the persistence of F418 *gfp* tr3 in pasteurised soil in the presence of host insects in 2009 and 2010 gave similar trends, although F418 *gfp* tr3 CFU recovery at 12 weeks was 348 % of the INIL in the 2009 experiment while it was 43,551 % of the INIL in the 2010 experiment. F418 *gfp* tr3 CFU recoveries in all treatments in pasteurised soil in the 2009 experiment were lower than in the 2010 experiment. Soil moisture content in pasteurised soil used for the 2009 experiment was 22 % GWC (equivalent to -0.85 bar) whereas soil water potential in pasteurised soil used for the 2010 experiment was -2 bar, so the soil was drier in 2010. The difference could be explained by the higher soil moisture used in 2009 as previous experiments presented in Chapter 4 showed that F418 *gfp* tr3 populations were higher in soils with lower moisture level.

F418 *gfp* tr3 CFU recovery in pasteurised soil with host insects was significantly higher than in non-sterile soil with host insects in both experiments (2009 and 2010). Conidiation on dead host insects was heavier in pasteurised soil compared to non-sterile soil. Groden & Lockwood (1991) found that a lower number of Colorado potato beetle *L. decemlineata* larvae supported sporulation following inoculation by *B. bassiana* (RS252) and incubation in Michigan soils compared to Rhode Island soils. Their study showed that Michigan soils were significantly more fungistatic than Rhode Island soils. Similarly in the present study, the level of conidiation on host insects in non-sterile soil is lower than in pasteurised soil indicating that the fungistatic activity in non-sterile soil probably had a negative impact on cadaver conidiation.

The presence of host arthropods is important for the persistence of entomopathogenic fungi, since entomopathogenic fungi are restricted to their host for multiplication in natural soils (Walstad *et al*., 1970). Keller *et al*. (2003) studied the distribution of *B. brongniartii* in soil in Switzerland and found that *B. brongniartii was* present only when its host insect

M. melolontha was present as well. Kessler *et al*. (2004) observed that *B. brongniartii* persistence decreased by 88 % in absence of host insect *M. melolontha* 16 month after application of *B. brongniartii* to meadows compared to a decline of 27 % on average for sites with host *M. melolontha*. The host insect used in the present experiment, *T. molitor*, is not a natural host for F418 since this insect is not present in pasture soil. However, similar results in terms of F418 *gfp* tr3 persistence in pasteurised and non-sterile soil would be expected if the target host, clover root weevil, had been used, as *S. lepidus* and *T. molitor* are of similar size and both support fungal conidiation on their cadavers. F418 is also pathogenic to Porina, *Wiseana* spp. (Appendix E) which is a common pest of New Zealand pastures (Barratt *et al*., 1990 cited by Brownbridge *et al*., 2008). Even though F418 is not a strong pathogen of Porina larvae (33 % mortality with 2×10^7 conidia/mL), this host could still contribute to increase F418 populations in pasture soil.

5.4.6 Conclusions

From the results presented in this chapter, the following conclusions can be made:

- F418 *gfp* tr3 persistence results in pasteurised soil in the presence of non-host or no insects were similar to results obtained in Chapter 3.
- Fluorescent microscopy showed that different F418 *gfp* tr3 propagules types (conidia, piece of mycelia) were present in pasteurised soil.
- Conidiation supported by *T. molitor* larvae in non-sterile soil was delayed and the level was inferior to conidiation supported by *T. molitor* larvae in pasteurised soil; therefore soil communities present in non-sterile soil might have a detrimental effect on conidiation.
- The use of the SSCP analysis showed a correlation between changes in F418 *gfp* tr3 persistence and changes in soil communities in pasteurised and non-sterile soil with host, non-host and no insect.
- F418 *gfp* tr3 could attach, germinate and produce appressoria on cuticle of live non-host *C. zealandica*.
- F418 *gfp* tr3 could pass through non-host *C. zealandica* guts and be fully recovered and viable. When F418 *gfp* tr3 ingested were not fully recovered, it was likely that conidia from faeces attached on cuticle of faeces covered *C. zealandica* larvae.

- The lower F418 *gfp* tr3 CFU recovery observed in pasteurised soil with non-host insects compared with no insects could be due to non-specific germination of F418 *gfp* tr3 conidia on the non-host insect cuticle, the presence of antagonists bacteria brought by the field collected non-host insects or a retention of viable conidia inside the non-host insect guts.

- The absence of difference in F418 *gfp* tr3 CFU recovery in non-sterile soil with non-host insects and without insects could be due to fungistasis preventing potential antagonistic bacteria brought by the non-host insects to degrade F418 *gfp* tr3 conidia or inhibit the attachment and germination on the non-host insects cuticle.

- Fungistasis present in non-sterile soil could prevent the loss of F418 *gfp* tr3 conidia observed in pasteurised soil, through lack of germination.

- The presence of host insects such as target hosts *S. lepidus* and non-target hosts (but also a pasture pest) *Wiseana* spp. is likely to help maintaining a high population of F418 in pasture soil via their potential infection by F418.

- The non-host pest grass grub *C. zealandica* could contribute to F418 dispersal in pastures as *C. zealandica* larvae can ingest and release viable F418 *gfp* tr3 conidia.

- The presence of non-host grass grub *C. zealandica* is likely to have no detrimental effect on F418 persistence in non-sterile soil, even though F418 *gfp* tr3 conidia can attach and germinate on *C. zealandica* cuticle in vitro and F418 *gfp* tr3 populations decrease in presence of *C. zealandica* in pasteurised soil.

Chapter 6
Beauveria bassiana F418 *gfp* tr3 persistence in soil in the presence of red clover plants

6.1 Introduction

In the previous chapters, the effect of different environmental factors on *B. bassiana* F418 and F418 *gfp* transformants populations was assessed in microcosms of pasteurised or non-sterile soil without plants. However *B. bassiana* F418 is a potential biocontrol agent against the pasture pest *S. lepidus* whose larva feed of clover roots (Gerard, 2001). Therefore, studying the population dynamics of *B. bassiana* F418 in soil in presence of clover plant is crucial; especially as plants can have an effect on entomopathogenic fungi populations (Hu & St. Leger, 2002; Bruck, 2005).

Hu & St. Leger (2002) found that *M. anisopliae* populations were maintained in the rhizosphere of cabbage *B. oleracea* plants whereas the populations in bulk soil declined, suggesting that the proximity of roots and root exudates was responsible for the rhizospheric effect observed. Following this finding, Bruck (2005) showed that *M. anisopliae* populations increased in the rhizosphere soil of Norway spruce *P. abies* and were significantly greater than in the bulk part of soil. McKinnon (2011) found that some strains of *B. bassiana* and *B. caledonica* could colonise the rhizosphere of *P. radiata* seedlings in non-sterile soil.

There are numerous reports of *B. bassiana* being an endophyte in plants. *Beauveria bassiana* can colonise the root, leaf or stem of a wide range of plant species such as corn (*Z. mays*), cocoa (*T. cacao*), coffee (*C. arabica*), date palm (*P. dactylifera*), opium poppy (*P. somniferum*), banana (*Musa spp.*), sorghum (*Sorghum sp.*), cotton (*G. hirsutum*), wheat (*T. aestivum*), bean (*P. vulgaris*), tomato (*L. esculentum*) and pumpkin (*Curcubita maxima*) (Bing & Lewis, 1991; Posada & Vega, 2005, 2006; Gomez-Vidal *et al*., 2006; Quesada-Moraga *et al*., 2006; Akello *et al.*, 2007; Tefera & Vidal, 2009 and Gurulingappa *et al.*, 2010) and can provide pest control as an endophyte (Bing & Lewis, 1991; Akello *et al.*, 2008). There is no report of *B. bassiana* being an endophyte in clover plant.

Entomopathogenic fungi have been shown to improve plant growth and health by controlling plant pathogens (Ownley *et al*., 2000, 2004 cited in Ownley *et al*., 2010) or mobilising nutrients that the plant cannot solubilise on their own (O'Brien 2009 cited by Fang & St. Leger 2010). The possibility of F418 and its *gfp* transformants colonising the clover rhizosphere or being an endophyte in clover plants, thus providing control of pest and pathogen and improving the plant growth, is a very appealing approach for the pasture system in New Zealand. However, further investigation is yet required.

The aim of this chapter was to determine if F418 *gfp* tr3 persistence was improved by the presence of red clover plants (*T. pratense*) and if in return red clover plants growth was higher in the presence of F418 *gfp* tr3. This aim was achieved by the following objectives (i) to test the effect of red clover plants on F418 *gfp* tr3 populations in the rhizosphere and bulk part of soil, (ii) to test the effect of the presence of F418 *gfp* tr3 on red clover plant growth and (iii) to test if F418 *gfp* tr3 is an endophyte in red clover plants. The effect of red clover plants on F418 *gfp* tr3 populations and vice versa will be tested in microcosms of pasteurised and non-sterile soil.

6.2 Material and methods

6.2.1 Evaluation of F418 *gfp* tr3 persistence in soil in the presence of clover

The objective of this experiment was to determine if F418 *gfp* tr3 persistence was improved in soil by the presence of red clover plant, in particular in the rhizosphere soil.

6.2.1.1 Microcosm

To assess the impact of the presence of red clover on F418 *gfp* tr3 populations in soil, 250 mL plastic containers (LabServ, NZ) were filled with 150 g of dry pasteurised soil or equivalent amount using non-sterile soil and moistened to -0.5 bar (Table 4.2). The pasteurised soil used was as described in section 3.2.1 and the non-sterile soil was collected in May 2010 and treated as described in the section 3.2.1.

F418 *gfp* tr3 conidia were produced and their viability checked using the protocol described in section 2.2.1.2. Soils were inoculated with 2 x 10^5 F418 *gfp* tr3 conidial suspension/g DWS for the treatment with fungus or were mixed with an equivalent volume of 0.01 % Triton® X-100 (BDH, Poole, UK) for the treatment without F418 *gfp* tr3.

Red clover (*T. pratense*) seeds (cultivar Sensation) were surface sterilised by immersion for 1 min in 70 % v/v ethanol followed by 20 min in approximately 0.5 % v/v sodium hypochlorite (10 % commercial bleach, Janola, NZ), then rinsed 6 times in SDW for 30 s each (Harris *et al*., 2006). Three red clover seeds were sown per container with clover treatment, in a triangular arrangement in the centre of the container at a depth of 5 mm.

The treatments were soil inoculated with F418 *gfp* tr3 prior to seed planting and clover, soil inoculated with F418 *gfp* tr3 and no plant and soil without F418 *gfp* tr3 and with clover. The three treatments were applied in pasteurised soil and non-sterile soil.

Containers were covered using sterile cellophane pie bags secured with rubber bands to avoid contamination. Extra clover plants were removed from the containers after three weeks to leave a single plant per container. There were 4 replicate containers per treatment and per sampling time. The containers were incubated at 19 °C with 16 h light/8 h dark using a split plot design (Fig. 6.1) and were watered 3 times a week to maintain the moisture at approximately -0.5 bar, determined by weight. The experiment was set up in May 2010.

Figure 6.1 Containers of pasteurised and non-sterile soil with or without F418 *gfp* tr3 in the presence or absence of red clover plants under growth light

6.2.1.2 Assessment of fungal persistence in bulk and rhizosphere soil

Containers were destructively sampled at 0, 3, 6, 9, 12, 15 and 18 weeks. For the treatments with clover, the plant was excised above the soil surface and kept aside and the remaining content of the container was poured into the cellophane bag (Fig. 6.2b and c). The root system was recovered from the bag and shaken briefly to remove loose soil (Fig. 6.2d) (Ahmad & Baker, 1987; Sivan & Harman, 1991). The roots with rhizosphere soil were placed in a pre-weighed 50 mL centrifuge tube (LabServ, NZ) and the weight was recorded. Soil in the cellophane pie bag was homogenised by hand then placed back in the original 250 mL container and further homogenised with a sterile spatula (Fig. 6.2e).

Approximately 20 g of soil was sampled and oven dried at 105 °C for 24 h for the determination of soil moisture.

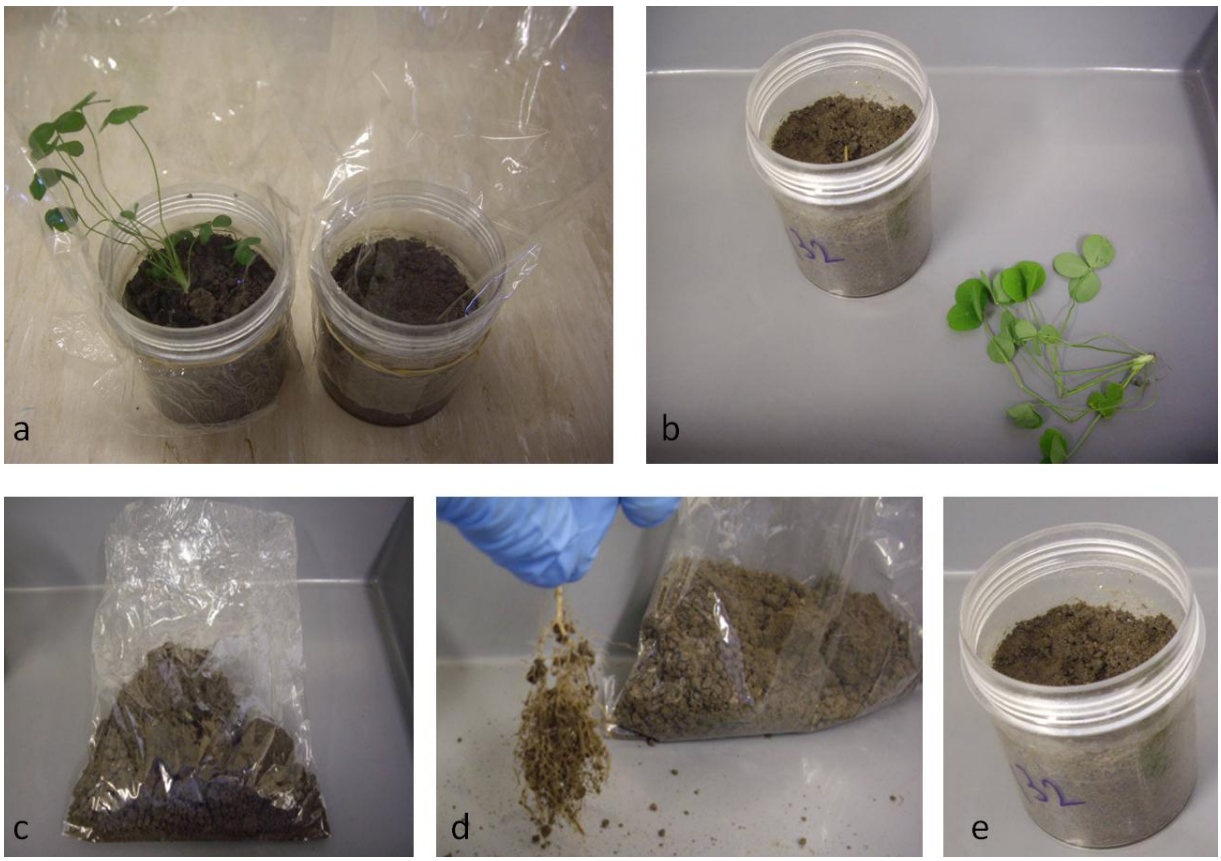

Figure 6.2 Process for sampling bulk and rhizosphere soil: a) Containers with a clover plant and without a plant, b) Above ground portion of clover plant excised for sampling, c) Total soil content container poured into the cellophane bag, d) Roots recovered from soil, e) Bulk soil homogenised in container before sampling.

For the estimation of F418 *gfp* tr3 persistence in bulk soil, 15 g of bulk soil from the container with homogenised soil above was sampled in a 250 screw cap container and 135 g of 0.01 % SDWA was added. The protocol was then as described for the soil dilution plating in section 3.2.4. Serial dilutions from 10^{-2} to 10^{-5} were prepared and 100 µL of each dilution spread onto a Petri plate of 10 % SDA+A+Hyg.

For the estimation of F418 *gfp* tr3 persistence in rhizosphere soil, 20 ml of 0.5 % Triton® X-100 was added to the 50 mL tubes containing the roots (Sivan & Harman, 1991). The tubes were sonicated for 3 min and vortexed to remove the rhizosphere soil from the roots and serial dilutions from 10^{-1} to 10^{-4} were prepared. One hundred µL of the dilutions were spread onto Petri plates of 10 % SDA+A+Hyg.

There were duplicate plates for each sample and dilution (bulk soil and rhizosphere soil). The plates were arranged in a RCBD and incubated at 20 °C in the dark for two weeks before counting the CFU. The persistence was expressed as CFU recovered/g DWS.

6.2.2 Effect of the presence of F418 *gfp* tr3 on clover plant growth

The objective of this experiment was to assess if the red clover growth could be improved by the presence of F418 *gfp* tr3 as shown by Ownley *et al.* (2000, 2004 cited in Ownley *et al.*, 2010) and O'Brien (2009 cited by Fang & St. Leger 2010). The top part of each clover plant, which was excised as described in section 6.2.1.2, was placed in a pre-weighed paper bag and weighed. Following removal of soil from the root system for the estimation of fungal persistence in the rhizosphere (6.2.1.2), the root system was placed between two layers of paper to absorb the 0.5 % Triton® X-100 and was then weighed and placed in the paper bag with its corresponding top plant part. Paper bags containing the clover plants (top plant and root system) were oven dried for at least 24 h at 105 °C before weighing the dry plant. Dry weight of plant was recorded for the sampling of 6, 9 and 12 weeks. Fresh weight of plant only was recorded for the sampling of 3, 15 and 18 weeks. At 3 weeks, the plant dry weight was too light to allow a precise weight reading.

6.2.3 Analysis for the endophytic presence of F418 *gfp* tr3 in clover

6.2.3.1 Analysis for the endophytic presence of F418 *gfp* tr3 by plating onto selective media

The objective of this experiment was to determine if F418 *gfp* tr3 could be an endophyte in red clover plant stem or roots.

Clover plants were assessed for endophytic F418 *gfp* tr3 at 15 and 18 weeks. Roots and stem were surface sterilised instead of oven drying as described in section 6.2.2. Surface sterilisation was done by immersion of the root system and stems into 95 % v/v ethanol for 30 s, followed by 4 min immersion in approximately 2 % sodium hypochlorite (50 % commercial bleach, Janola, NZ), and rinsing 3 times in SDW for 1 min each (Sturz *et al*., 1997). The roots and stems were cut in approximately 2 cm long pieces and 5 to 10 random pieces of root or stem were plated onto 10 % SDA+A+Hyg. There were duplicate plates for stem pieces and duplicate plates for root pieces for each clover plant (Fig. 6.3). The plates were incubated at 20 °C for 3 weeks before observing for endophytic F418 *gfp* tr3 growth out of the roots or stem. All the clover plants grown in soil with F418 *gfp* tr3 and without fungus were assessed for endophytic F418 *gfp* tr3; clover plants from soil without fungus were used as negative control.

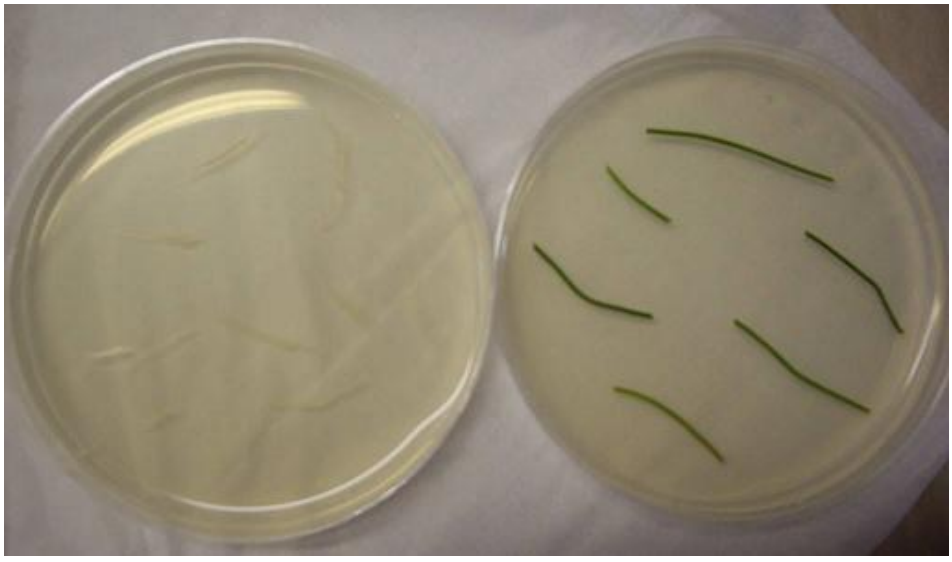

Figure 6.3 Surface sterilised clover roots and stems pieces onto selective media for the detection of endophytic F418 *gfp* tr3.

6.2.3.2 Analysis of endophytic F418 *gfp* tr3 using PCR

The objective of this experiment was to assess if the F418 *gfp* tr3 endophytic activity in red clover plant could be confirmed using molecular tools. Following the assessment for F418 *gfp* tr3 presence as an endophyte using selective media described in section 6.2.3.1, pieces of surface sterilised root and stem were stored in 1.5 mL tubes at -80 °C until DNA extraction. Approximately 5 pieces of root and 5 pieces of stem were stored for each plant with stems and roots kept in separate tubes.

DNA from roots and stems was extracted using DNeasy plant mini kit (Qiagen, Netherlands) according to manufacturer's instructions. Prior to extraction, pieces of root and stem were ground using a sterile plastic pestle in a 1.5 mL tube dipped in liquid nitrogen. Pieces of roots could not be ground thoroughly because of their texture. DNA quality was assessed by gel electrophoresis. Each DNA sample (10 µL) was run with 2 µL of loading dye alongside a 1kb+ DNA ladder (Invitrogen) in a 1 % agarose gel; gel running and visualisation are described in section 2.2.3.2. DNA concentration was assessed by spectrophotometry (NanoDrop, Thermo scientific, USA).

The presence or absence of F418 *gfp* tr3 was assessed by amplifying a fragment from the *gfp* gene and using primers designed to amplify a fragment from the chalcone reductase, from clover, as a positive control. DNA amplification of the chalcone reductase region of clover was performed using CHR-CDS_F 5' AACAACATGGGTAGTGTTGAAA 3' and CHR-CDS_R 5' TTATATTTCATCATCCCATAGGTCA 3' (B. Franzmayr, personal communication). DNA amplification of the *gfp* region was performed using GFP ID_F 5' ACGTAAACGGCCACAAGTTC 3' and GFP ID_R 5' TGCTCAGGTAGTGGTTGTCG 3'.

Each PCR contained 1 × PCR reaction buffer with 1.5 mM $MgCl_2$ (Roche, Basel, Switzerland), 200 µM of each dNTP (Roche), 10 pM of each primer (Invitrogen, Life Technologies, CA, USA), 1.25 U Taq DNA polymerase (Roche) and 10 ng of isolated DNA in a total volume of 50 µL. A negative, DNA-free control was included in every PCR run to ensure absence of contamination. A positive control for the *gfp* gene primers was included using DNA extracted from *B. bassiana* F418 *gfp* tr3. A Veriti 96 well thermal cycler (AB Applied Biosystems, Life Technologies, CA, USA) was used and the temperature profile for the clover chalcone reductase primers was 2 min at 95 ºC followed by 40 cycles of 15 s at 94 ºC, 30 s at 48 ºC and 2 min at 68 ºC. The 40 cycles were followed by 5 min at 68 ºC. The temperature profile for the *gfp* gene primers was 2 min at 94 ºC followed by 40 cycles of 30 s at 94 ºC, 30 s at 52 ºC and 1 min at 72 ºC. The 40 cycles were followed by 10 min at 72 ºC. Amplified products were visualised as described in section 2.2.3.2.

The DNA detection threshold for the *gfp* gene primers was assessed by amplifying the *gfp* gene region (using GFP ID_F and GFP ID_R as described above) from a serial dilution of F418 *gfp* tr3 DNA. F418 *gfp* tr3 DNA was diluted to 5 ng/µL, 1 ng/ µL, 0.5 ng/ µL, 0.1 ng/ µL, 50 pg/ µL, 10 pg/ µL, 5 pg/ µL and 1 pg/ µL. Each PCR contained 1 × PCR reaction buffer with 1.5 mM $MgCl_2$ (Roche, Basel, Switzerland), 200 µM of each dNTP (Roche, Basel, Switzerland), 5 pM of each primer (Invitrogen, Life Technologies, CA, USA) and 0.625 U Taq DNA polymerase (Roche, Basel, Switzerland) and 1 µL of F418 *gfp* tr3 from the serial dilution in a total volume of 25 µL. A negative, DNA-free control was included in every PCR run to ensure absence of contamination. A Veriti 96 well thermal cycler (AB Applied Biosystems) was used and the temperature profile for the *gfp* gene primers was used as described in the above paragraph. Amplified products were visualised as described in section 2.2.3.2.

6.2.4 Statistical analysis

All CFU recovery data and plant weights were $\log_{10}$ transformed to achieve a normal distribution essential to the use of ANOVA (Olsen, 2003). A split plot ANOVA was carried out, with treatment factors being the treatments (soil type, plant, fungus, soil part) and time, with a linear polynomial contrast specified where appropriate.

Significance was evaluated at $P < 0.05$ for all analyses and mean separation was executed by the unprotected LSD test. All data analyses were performed using the statistical software Genstat version 12.2 (VSN International). All statistical results tables are presented in Appendix M.

6.3 Results

6.3.1 Effect of the presence of clover on persistence of F418 *gfp* tr3 in soil

6.3.1.1 Effect of the presence or absence of clover on F418 *gfp* tr3 in total soil

The effect of presence or absence of clover plant and soil type (pasteurised and non-sterile soil) on F418 *gfp* tr3 persistence in total soil (bulk plus rhizosphere soil) was assessed over 18 weeks (Table 6.1 and Fig 6.4). F418 *gfp* tr3 populations in pasteurised soil with and without clover increased significantly during the initial 3 weeks of the experiment and were maintained significantly higher than the initial inoculum level (INIL) between 3 and 18 weeks and were 786 % of the INIL in pasteurised soil with clover and 936 % of the INIL in pasteurised soil without plant. There was no difference in F418 *gfp* tr3 populations between pasteurised soil with clover and without clover during the whole experiment.

F418 *gfp* tr3 populations in non-sterile soil with and without clover plants were not significantly different from the INIL at all sampling times. There was no difference in F418 *gfp* tr3 populations between non-sterile soil with clover and without clover during the whole experiment.

F418 *gfp* tr3 populations in pasteurised soil with and without clover were significantly higher (from 10 to 26 fold) than in non-sterile soil with and without clover between 3 and 18 weeks.

Table 6.1 F418 *gfp* tr3 populations recovered (CFU/g DWS and log_{10} value) in pasteurised and non-sterile soil with clover or without plant over 18 weeks.

CFU/g DWS **(log_{10})**	Week 0	Week 3	Week 6	Week 9	Week 12	Week 15	Week 18
Pasteurised soil + clover	1.40×10^5 **(5.15)**	1.80×10^6 **(6.26)**	1.98×10^6 **(6.30)**	1.41×10^6 **(6.15)**	1.86×10^6 **(6.27)**	2.63×10^6 **(6.42)**	1.10×10^6 **(6.04)**
Pasteurised soil no plant	1.40×10^5 **(5.15)**	2.45×10^6 **(6.39)**	2.18×10^6 **(6.34)**	1.66×10^6 **(6.22)**	2.12×10^6 **(6.33)**	1.74×10^6 **(6.24)**	1.31×10^6 **(6.12)**
Non-sterile soil + clover	9.75×10^4 **(4.99)**	1.19×10^5 **(5.08)**	1.12×10^5 **(5.05)**	9.64×10^4 **(4.98)**	8.59×10^4 **(4.93)**	6.92×10^4 **(4.84)**	7.43×10^4 **(4.87)**
Non-sterile soil no plant	9.75×10^4 **(4.99)**	1.10×10^5 **(5.04)**	9.51×10^4 **(4.98)**	9.84×10^4 **(4.99)**	9.91×10^4 **(5.00)**	8.71×10^4 **(4.94)**	9.16×10^4 **(4.96)**
LSD ($P < 0.05$)				**0.37**			

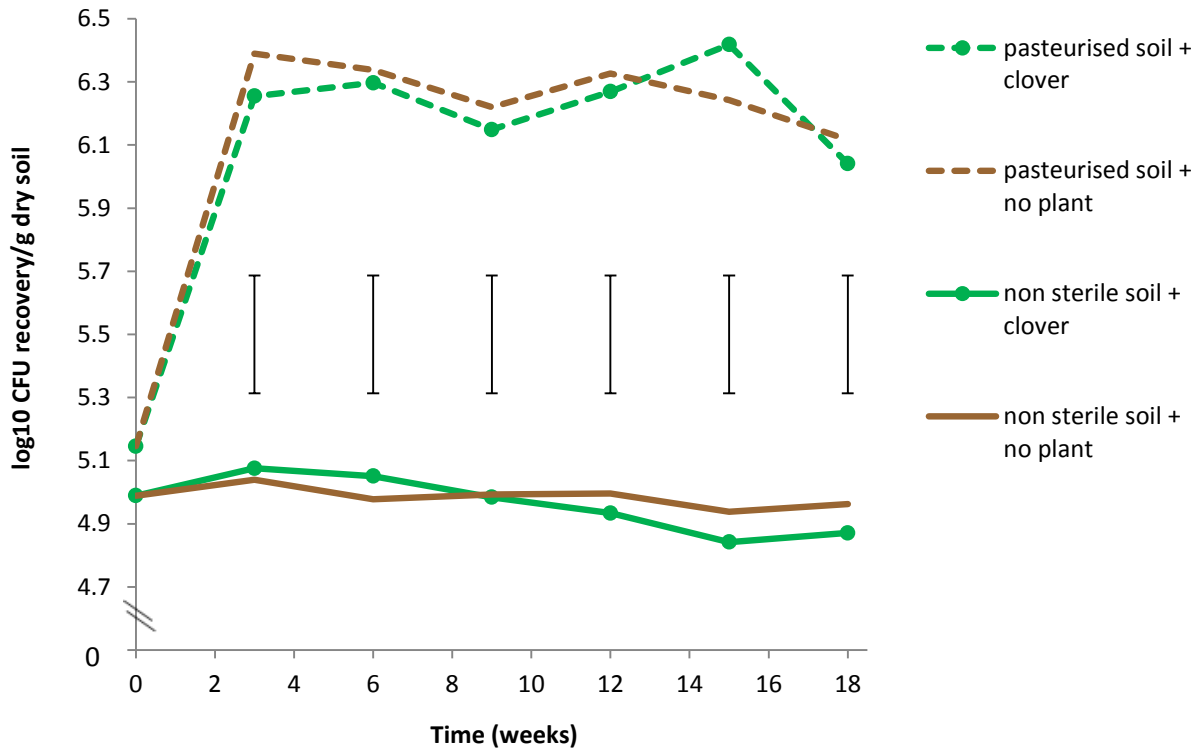

Figure 6.4 F418 *gfp* tr3 populations recovered (log_{10} CFU/g DWS) from total soil (bulk and rhizosphere soil) (pasteurised and non-sterile) with and without clover plant in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

6.3.1.2 Effect of the presence of clover plant on F418 *gfp* tr3 in bulk and rhizosphere soil

The effect of clover plant and soil type (pasteurised and non-sterile soil) on F418 *gfp* tr3 persistence in bulk soil versus rhizosphere soil was assessed over 18 weeks (Table 6.2 and Fig 6.5). F418 *gfp* tr3 populations in bulk pasteurised soil increased significantly during the initial 3 weeks of the experiment and were maintained significantly higher than the INIL between 3 and 18 weeks; F418 *gfp* tr3 populations were 600 % of the INIL at 18 weeks, although there was a significant decrease between 15 and 18 weeks. F418 *gfp* tr3 populations in rhizosphere pasteurised soil increased significantly compared to the INIL until 9 weeks when F418 *gfp* tr3 populations were a 100 fold the INIL. Then, F418 *gfp* tr3 populations in rhizosphere pasteurised soil decreased significantly between 9 and 12 weeks, although F418 *gfp* tr3 populations were still significantly higher than the INIL followed by a stabilisation between 12 and 18 and 4,531 % of the INIL recovered at 18 weeks. F418 *gfp* tr3 populations in the rhizosphere part of pasteurised soil were significantly higher than in the bulk part per g of DWS at 6, 9 and 18 weeks.

F418 *gfp* tr3 populations in the bulk and rhizosphere part of the non-sterile soil were not significantly different from the INIL at all sampling times and the F418 *gfp* tr3 populations in the bulk and rhizosphere part were not different from each other. F418 *gfp* tr3 populations in the bulk and rhizosphere part of the non-sterile soil were significantly lower than in the bulk and rhizospheric part of the pasteurised soil between 3 and 18 weeks.

Table 6.2 F418 *gfp* tr3 populations recovered (CFU/g DWS and $\log_{10}$ value) from the rhizosphere and bulk part of pasteurised and non-sterile soil with clover plant over 18 weeks.

CFU/g DWS **($\log_{10}$)**	Week 0	Week 3	Week 6	Week 9	Week 12	Week 15	Week 18
Pasteurised soil rhizosphere	1.40×10^5 **(5.15)**	1.90×10^6 **(6.28)**	6.85×10^6 **(6.84)**	1.41×10^7 **(7.15)**	3.52×10^6 **(6.55)**	5.30×10^6 **(6.72)**	6.34×10^6 **(6.80)**
Pasteurised soil bulk	1.40×10^5 **(5.15)**	1.80×10^6 **(6.26)**	1.95×10^6 **(6.29)**	1.38×10^6 **(6.14)**	1.71×10^6 **(6.23)**	2.46×10^6 **(6.39)**	8.39×10^5 **(5.92)**
Non-sterile soil rhizosphere	9.75×10^4 **(4.99)**	1.90×10^5 **(5.28)**	1.17×10^5 **(5.07)**	1.21×10^5 **(5.08)**	3.58×10^4 **(4.55)**	1.09×10^5 **(5.04)**	5.40×10^4 **(4.73)**
Non-sterile soil bulk	9.75×10^4 **(4.99)**	1.19×10^5 **(5.08)**	1.11×10^5 **(5.04)**	9.59×10^4 **(4.98)**	9.02×10^4 **(4.96)**	6.61×10^4 **(4.82)**	7.60×10^4 **(4.88)**
LSD ($P < 0.05$)				**0.46**			

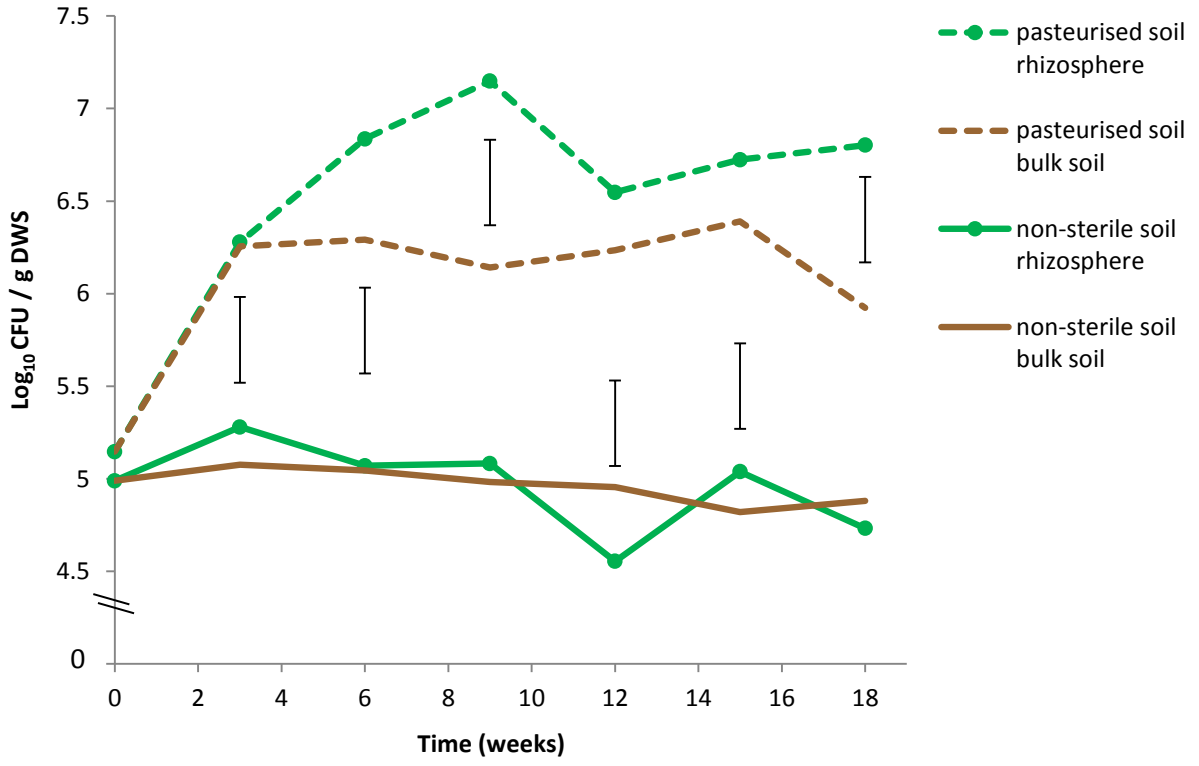

Figure 6.5 F418 *gfp* tr3 populations recovered ($\log_{10}$ CFU/g DWS) from bulk and rhizosphere part of pasteurised and non-sterile soil with clover plant in laboratory microcosms. Vertical bars are LSD ($P < 0.05$).

6.3.2 Effect of F418 *gfp* tr3 on clover plant growth

The effect of the presence of F418 *gfp* tr3 on the clover plants total dry weight (at 6, 9 and 12 weeks) and fresh weight (at 3, 15 and 18 weeks) was assessed in pasteurised and non-sterile soil (Table 6.3). There was no significant difference in clover plant weight between the different treatments at 3, 9, 12, 15 and 18 weeks. The only difference was the plant dry weight of the clover grown in pasteurised soil inoculated with F418 *gfp* tr3 was significantly lower than the clover grown in non-sterile soil inoculated with F418 *gfp* tr3 after the 6 weeks.

Table 6.3 Clover plant dry or fresh weight (mg and $\log_{10}$ of mg) grown in pasteurised and non-sterile soil in the presence or absence of F418 *gfp* tr3 at different sampling times. LSD ($P < 0.05$) is given for the comparison of the weight in $\log_{10}$ mg.

Treatment	Plant weight	Sampling time (weeks)					
	(mg)	3	6	9	12	15	18
Pasteurised soil with	Dry weight		5.2	24.0	89.1		
F418 *gfp* tr3	Fresh weight	29.5				574.1	668.3
	$\log_{10}$ weight	**1.47**	**0.72**	**1.39**	**1.95**	**2.76**	**2.83**
Pasteurised soil	Dry weight		7.1	30.9	67.0		
without F418 *gfp* tr3	Fresh weight	27.2				687.1	527.2
	$\log_{10}$ weight	**1.43**	**0.85**	**1.49**	**1.83**	**2.84**	**2.72**
Non-sterile soil with	Dry weight		11.3	24.0	78.0		
F418 *gfp* tr3	Fresh weight	25.9				796.2	736.2
	$\log_{10}$ weight	**1.41**	**1.05**	**1.39**	**1.89**	**2.90**	**2.87**
Non-sterile soil	Dry weight		6.1	29.5	83.4		
without F418 *gfp* tr3	Fresh weight	28.7				660.7	918.3
	$\log_{10}$ weight	**1.46**	**0.78**	**1.47**	**1.92**	**2.82**	**2.96**
LSD ($P < 0.05$)				**0.32**			

6.3.3 Investigation of F418 *gfp* tr3 as an endophyte in clover

6.3.3.1 Detection of endophytic F418 *gfp* tr3 on selective media

None of the pieces of roots and stems plated from the 15 week sampling showed F418 *gfp* tr3 growth (Fig. 6.6a and b). From the 18 week sampling, two plates with pieces of roots from two different clover plants both grown in pasteurised soil showed colonies characteristic

of F418 *gfp* tr3 (Fig. 6.6c) and one plate with pieces of stem from a clover plant grown in non-sterile soil showed colonies characteristic of F418 *gfp* tr3 (Fig. 6.6d). These colonies were growing from the cut end of the stems and roots, indicative of endophyte recovery.

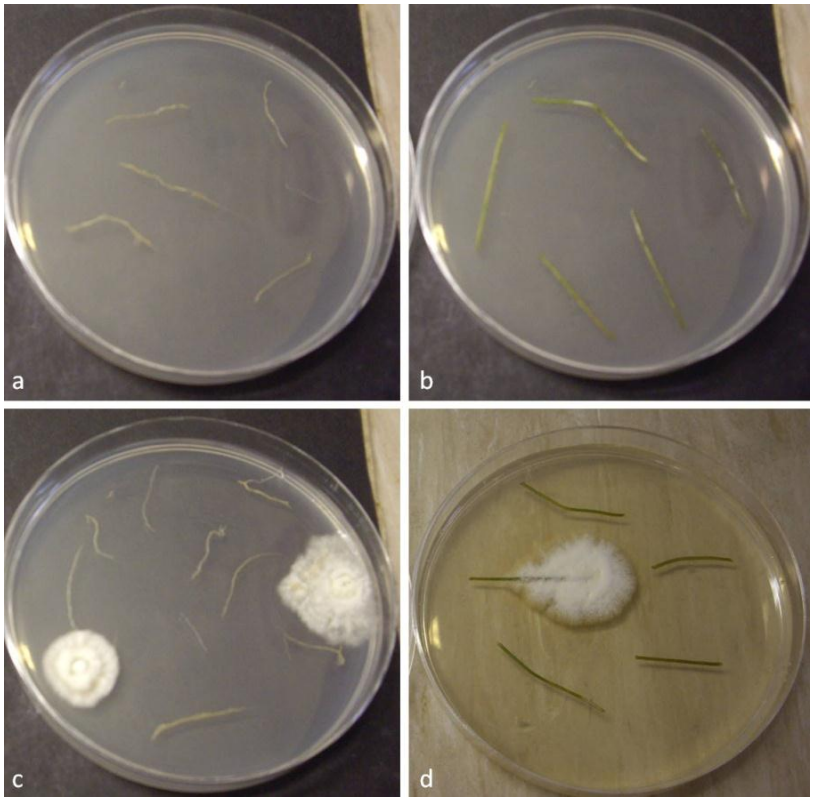

Figure 6.6 Photographs of surface sterilised pieces of clover roots and stems plated onto selective media for F418 *gfp* tr3 endophyte investigation. a) Root pieces with no fungal growth. b) Stem pieces with no fungal growth. c) Some root pieces with colonies characteristic of F418 *gfp* tr3 from clover plant grown in pasteurised soil. d) Piece of stem with a colony characteristic of F418 *gfp* tr3 from a clover plant grown in non-sterile soil.

6.3.3.2 Detection of endophytic F418 *gfp* tr3 using PCR

DNA quality was checked by electrophoresis (Fig. 6.7) and it showed that DNA extracted from the clover roots was in very low quantity (Fig. 6.7a) or the quality was poor while the DNA extracted from the clover stem (Fig. 6.7b) was of good quality for most samples. The DNA concentration was assessed using spectrophotometry and DNA concentration from clover root samples ranged between 2.04 and 5.94 ng/µL with an average of 4.29 ng/µL. DNA concentration from clover stem samples ranged between 2.88 and 9.75 ng/µL with an average of 5.07 ng/µL.

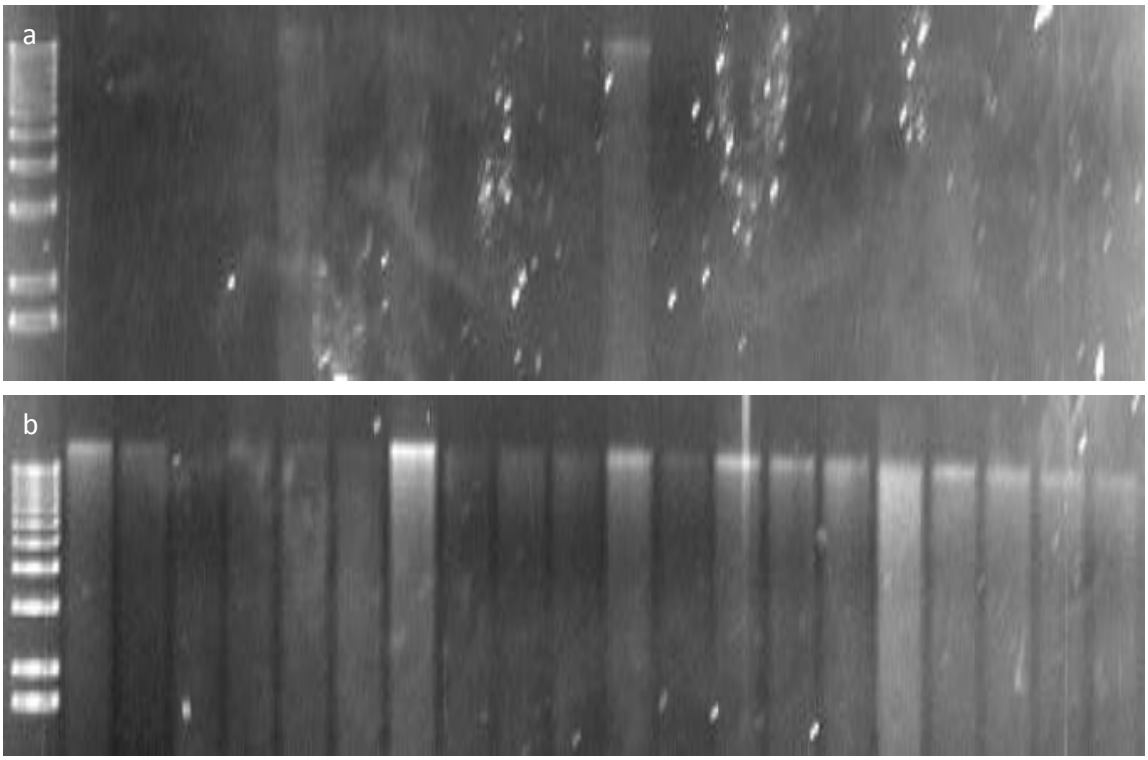

Figure 6.7 1 % agarose gels for quality check of the DNA extracted from (a) clover roots and (b) stems from 10 µL of DNA alongside a 1 kb plus DNA ladder (first lane on each gel)

It was possible to amplify the chalcone reductase gene from all DNA extracted from stems samples (Fig. 6.8a) whereas it was not possible for some DNA extracted from roots samples and for the samples which did amplify the clover gene, most of the bands obtained were weak. PCRs for the *gfp* gene did not amplify bands from any of the DNA extracted from the root and stem samples (Fig. 6.8b), even the samples from the clover plants whose pieces of root and stem gave rise to F418 *gfp* tr3 colonies on selective media (6.3.3.1).The control for the *gfp* gene using F418 *gfp* tr3 DNA was positive on lane 33 (Fig. 6.8b). Negative controls without DNA (lane 34) did not have any bands (Fig. 6.8a and b).

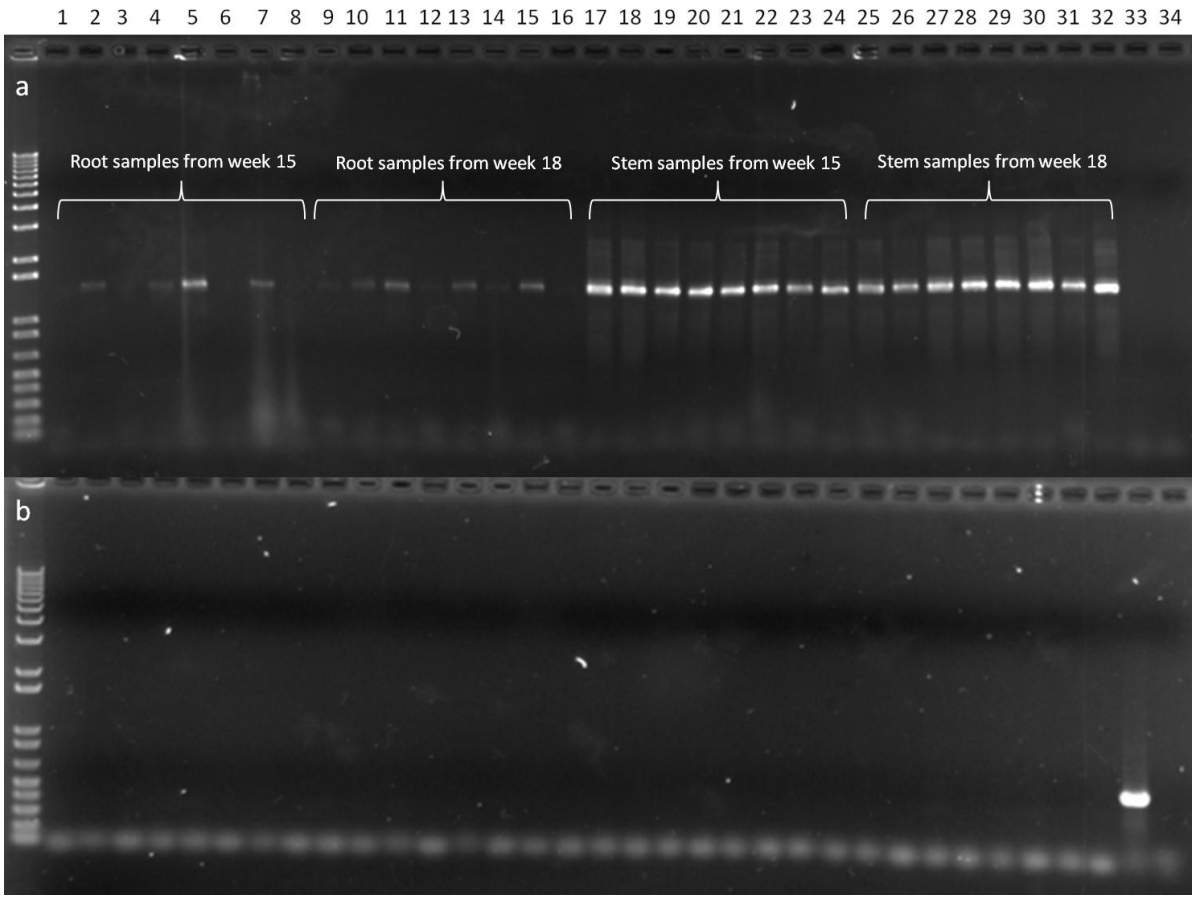

Figure 6.8 1 % agarose gels for the molecular analysis of F418 *gfp* tr3 endophytic presence in clover root and stem sampled at 15 and 18 weeks: a) Gel fom the amplification of the 1,500 bp chalcone reductase gene, b) Gel from the amplification of the 400 bp *gfp* gene with the positive control using F418 *gfp* tr3 DNA on lane 33. The first line on each gel was the 1 kb plus DNA ladder. The pieces of root which gave rise to F418 *gfp* tr3 colonies were in lanes 14 and 15. The piece of stem which gave rise to a F418 *gfp* tr3 colony was in lane 26.

The detection threshold for the amplification of the *gfp* gene from F418 *gfp* tr3 DNA using the GFP ID primer set was 50 pg of F418 *gfp* tr3 DNA per 25 µL PCR reaction using pure DNA (Fig 6.9). With 50 pg of F418 *gfp* tr3 DNA per 25 µL PCR reaction, the band obtained was very faint. The band was clearer with 0.1 ng of F418 *gfp* tr3 DNA per 25 µL PCR reaction.

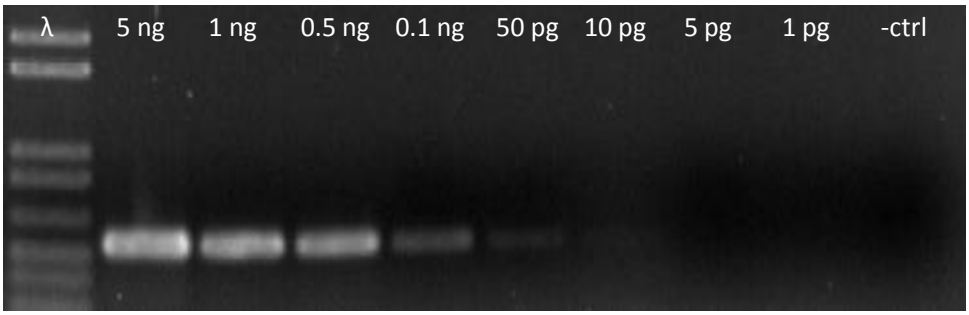

Figure 6.9 1 % agarose gel for the detection of the threshold of F418 *gfp* tr3 DNA needed using GFP ID primer set to obtain amplification of the *gfp* gene with a serial dilution of F418 *gfp* tr3 DNA from 5 ng/µL to 1 pg/µL in a 25 µL volume PCR reaction. λ is the 1 kb plus DNA ladder. –ctrl is the DNA-free negative control.

6.4 Discussion

6.4.1 Impact of clover plant presence on F418 *gfp* tr3 populations in soil

This study showed that F418 *gfp* tr3 populations were higher in the rhizosphere of red clover plants compared to the bulk pasteurised soil. McKinnon (2011) found that populations of *B. bassiana* and *B. caledonica* isolates were greater in the rhizosphere soil of *P. radiata* compared to the bulk soil in non-sterile soil. Hu & St. Leger (2002) and Bruck (2005) showed that populations of *M. anisopliae* were greater in the rhizosphere soil of cabbages plant and Norway spruce, respectively, compared to the bulk part of soil. Hu & St. Leger (2002) and Bruck (2005) concluded that *M. anisopliae* strains used in their study were rhizosphere competent. Similarly, in the present study, F418 *gfp* tr3 showed rhizosphere colonisation with red clover plant in pasteurised soil. Lingg & Donaldson (1981) showed growth of *B*. *bassiana* in sterile soil amended with a carbon and nitrogen source. Root exudates can provide nutrient to fungus which could explain the higher fungal population in the rhizosphere soil compared to the bulk soil in pasteurised soil. Wyrebek *et al*., (2011) found a specific association between different species of *Metarhizium* and different species of plants in Canada while studying plant's rhizosphere and showed that *M. robertsii* conidia germinated better than *M. brunneum* and *M. guizhouense* in *Panicum virgatum* L. (Poales: Poaceae) root exudates. Simiarly, Fisher *et al*. (2011) also found some species of *Metarhizium* and *Beauveria* are significantly associated with specific plants in the

rhizopshere such as *B. bassiana* Bbas-16 was found significantly associated with grapes. Wang *et al*. (2011) found that *Metarhizium robertsii*'s ability to adhere to plant roots contribute to maintain the population size in the field and showed the importance of the relationship with plants.

However, there was no difference in F418 *gfp* tr3 populations between rhizosphere of clover plant and bulk soil in non-sterile soil during this 18 week experiment. Bruck (2005) found significant differences in *M. anisopliae* populations in the rhizosphere and bulk soil between 29 and 49 weeks after the start of the experiment, but did not monitor the populations in the rhizosphere before 29 weeks; therefore it is not known when differentiation between populations and bulk soil began. Hu & St. Leger (2002) monitored the *M. anisopliae* populations in the rhizosphere and bulk soil for 43 weeks and found a significant difference between rhizosphere and bulk soil populations at all samplings from 1 month. McKinnon (2011) found differences in *Beauveria* sp. populations between bulk and rhizosphere soil of pine trees, *Brassica* sp., celery (*Apium graveolens*, L., Apiales: Apiaceae) and onions (*Allium cepa,* L., Asparagales: Amaryllidaceae) within less than 12 weeks.

In the present study, F418 *gfp* tr3 populations were monitored for 18 weeks which was shorter than studies from Hu & St. Leger (2002) and Bruck (2005) but longer than McKinnon (2011) experiments. Also, cabbage roots growth is much faster than clover roots. Stoffella & Fleming (1990) obtained an average of 32.3 g of fresh cabbage roots after 23 weeks of growth whereas in the present study, the average clover roots fresh weight was 117 mg after 18 weeks of growth. Therefore, F418 *gfp* tr3 populations in non-sterile soil with red clover plant did not show rhizosphere colonisation during this 18 week experiment, but a general conclusion about F418 *gfp* tr3 not having rhizosphere colonising ability in non-sterile soil with red clover plants cannot be drawn considering the shorter experimental time compared to Hu & St. Leger (2002) and Bruck (2005) and the clover plant growth rate being much slower than cabbage plants. The choice of an 18 week-long experiment was mostly to be consistent with the average duration of all the previous experiment as well as the limited space to run such an experiment in PC2 (physical containment level 2) conditions due to the use of the *gfp* transformant.

In this study, F418 *gfp* tr3 populations in the total soil (bulk + rhizosphere soil) were not different in the presence or absence of clover plants. Bulk soil formed 96 % of the total soil, therefore even when F418 *gfp* tr3 populations were higher in the rhizosphere pasteurised soil, it could not be detected in the total amount of soil.

F418 *gfp* tr3 populations increased significantly in both rhizosphere and bulk pasteurised soil and remained greater than the initial level during the whole experiment. This finding is

consistent with F418 *gfp* tr3 persistence results from Chapters 4 and 5 in pasteurised soil. Similarly, F418 *gfp* tr3 populations in non-sterile soil (both in rhizosphere and bulk soil) were never significantly different from the INIL, as observed in Chapter 5 in non-sterile soil. Hu & St. Leger (2002) showed that *M. anisopliae* populations (GMa and GPMa wild-type and transformant of strain ARSEF1080) were maintained at 10^5 propagules/g soil whereas fungal population in bulk soil declined from 10^5 to 10^3 propagules/g soil 6 months after application. Similarly to Hu & St. Leger, F418 *gfp* tr3 populations' levels were maintained in rhizosphere non-sterile soil. In the present study, fungal populations in non-sterile bulk soil were similar to populations in rhizospheric soil and were maintained at initial levels throughout the experiment. The present experiment was carried in non-sterile soil under laboratory conditions which may be a less stressful environment than the field and might explain why fungal populations were maintained in bulk soil. Bruck (2005) showed that *M. anisopliae* populations (strain F52) increased in peat and bark-base potting mix and that the population in the rhizosphere was greater than in bulk part of the media. Considering that horticultural potting media are often microbially impoverished because of the acidic peat content (Bruck, 2005), it is not surprising that the present findings in pasteurised soil (which is microbiologically poor but not entirely sterile) are quite similar to Bruck's findings.

6.4.2 Impact of presence of F418 *gfp* tr3 on clover plant growth

The presence of F418 *gfp* tr3 in soil did not have any effect on red clover plant growth in general during the 18 week experiment. The only significant difference in plant weight was between clover plants grown in presence of F418 *gfp* tr3 in pasteurised soil and non-sterile soil at 6 weeks with dry weight of plants grown in pasteurised soil being lighter. However, there were no differences in plant weights when grown in presence of F418 *gfp* tr3 in pasteurised soil compared to non-sterile soil at any other sampling times. Therefore, the difference observed could not be explained by the soil type.

One hypothesis to attempt to explain this difference could be the lack of homogeneity of the light source. One growth light was available only and it was not possible to homogenise the light source over all the plants; therefore there was a noticeable difference in plant size between clover plants situated just under the growth light source and the ones placed at either extremity of the light source. This difference was also supported by statistics as plants grown in block 3 (under the light) were significantly heavier ($P < 0.05$) than plants grown in block 4 situated at the extremity of the light source. A complete randomized block design

was used inside each sampling time plot to minimize the impact of the lack of homogeneity of the light source; however at 6 weeks, two of the four replicates of the treatment with clover plant in pasteurised soil with F418 *gfp* tr3 were situated on the extremities of the light range whereas all the replicates of the treatment with clover plant in non-sterile soil with F418 *gfp* tr3 were situated under the light source. Therefore, the difference in plant weights observed at 6 weeks was likely due to the position of the plant under and further from the light source. Th effect of F418 *gfp* tr3 on clover plant growth was tested because it has been shown that entomopathogenic fungi can improve plant growth as endophyte. Sasan & Bidochka (2012) showed that *M. robertsii* present as an endophyte in switchgrass *P. virgatum* and haricot beans *P. vulgaris* led to an increase in plant root hairs and a faster growth compared to the untreated controls.

6.4.3 F418 *gfp* tr3 as an endophyte in clover plant

The present study showed that F418 *gfp* tr3 can be an endophyte in red clover plants. F418 *gfp* tr3 colonies developed from root and stem pieces of red clover grown in both pasteurised and non-sterile soil. The efficiency of the surface sterilisation method was not tested as by Reay *et al.* (2010); however the surface sterilisation protocol used was a published method (Sturz *et al.*, 1997) and all the F418 *gfp* tr3 colonies grew from the tip of the cut pieces of clover plant. The roots and stems pieces of red clover plants were placed onto 10 % SDA+A+Hyg media, inhibiting the growth of any organisms other than those resistant to hygromycin. Therefore, the fungal colonies which were observed growing from the cut ends of pieces of clover roots and stems could be confirmed as endophytic F418 *gfp* tr3. This finding means that F418 could potentially have a better persistence in pasture as an endophyte in clover plants.

Molecular techniques did not confirm the presence of F418 *gfp* tr3 as an endophyte of red clover plants. The detection threshold of F418 *gfp* tr3 DNA was 50 pg in a 25 µL PCR reaction. The average C value of haploid cells in fungi from the Hypocreales order (to which *B. bassiana* belongs) is 0.038 pg (Kullman *et al*., 2005); therefore, the detection threshold of the PCR used was approximately 1,300 F418 *gfp* tr3 nuclei in a 25 µL PCR reaction. The high detection threshold of the PCR method used was likely responsible for the lack of *gfp* gene amplification from root and stem samples from the clover plant which had been confirmed to have endophytic F418 *gfp* tr3 by the plating culture method.

Also, there was a poor DNA quality extracted from root samples. Bright amplimers for the clover chalcone-reductase gene were obtained from only half of the root DNA samples whereas all stem DNA samples amplified the chalcone-reductase gene with bright amplimers obtained. The poor DNA quality extracted from roots was also obvious when the DNA was separated on 1 % agarose gel.

The poor DNA quality was probably due to the DNA extraction method used. Root pieces were ground using a plastic pestle in a 1.5 mL tube dipped in liquid nitrogen. Root tissue was hard and the grinding was difficult. A ceramic mortar and pestle should have been used to grind root pieces in liquid nitrogen. The DNA extraction method used did however work for softer plant tissue such as clover stem.

The combination of the plant DNA extraction method and the PCR method were likely to be responsible for the lack of confirmation of the presence of F418 *gfp* tr3 in clover root and stem pieces. A more appropriate DNA extraction method should have been used and a molecular technique with a lower DNA detection level such as nested PCR or quantitative PCR should have been used (Enkerli & Widmer, 2010). Unfortunately, all clover part samples were used for the DNA extraction and therefore, DNA extraction using a better approach could not be carried out.

6.4.4 Use of red clover

Red clover (*T. pratense*) was used in this study. Hardwick (1998) showed that adults clover root weevil prefer to feed on excised leaves of white clover (*T. repens*) over red clover in a choice and non-choice situation but had no preferences with red and white clover seedlings. Hackell & Gerard (2004) showed that CRW larvae do not have any feeding preferences between red or white clover nodules. However both studies by Hardwick (1998) and Hackell & Gerard (2004) were done under laboratory conditions. Watson *et al.* (2002) studied the plant damage and CRW number/plant in a field trials with white and red clover and found that CRW were more present on white clover plant and the damage was also higher than on red clover plant. The choice of using red clover in this study was primarily driven by the faster growth and larger root system obtained with red clover compared to white clover. The timeframe for the experiment was limited and fast growing clover species was essential to be able to sample rhizosphere soil. It would have been interesting to repeat this experiment using white clover as white clover covers higher superficies than red clover in perennial

pastures in New Zealand (Rolston P., personal communication) and is more sensitive to CRW than red clover (Watson *et al*., 2002).

6.4.5 Conclusions

From the results presented in this chapter, the following conclusions can be made:

- F418 *gfp* tr3 showed rhizosphere colonisation in the presence of red clover plants in pasteurised soil, whereas F418 *gfp* tr3 persistence was not improved in non-sterile soil in the presence of red clover plant.
- F418 *gfp* tr3 populations increased with all treatments in pasteurised soil whereas F418 *gfp* tr3 populations were not different from the initial inoculum level in all non-sterile soil treatments.
- Red clover plant growth was not improved by the presence of F418 *gfp* tr3 nor by the type of soil.
- F418 *gfp* tr3 was found to be an endophyte in the roots and stems of red clover plants grown in both pasteurised and non-sterile soil using a culture plating method; however the PCR failed to confirm the findings.
- Persistence of F418 in pastures could potentially be improved by the endophytic properties in clover plants.

Chapter 7
General discussion

This PhD study was undertaken with the aim of gaining a better understanding of soil environmental factors affecting the persistence of *B. bassiana* F418 (aided by the use of *gfp* transformants) in order to improve the use of this biocontrol agent against the clover root weevil. This final chapter will attempt to synthetise all the findings made in each chapter and present the novelties of this research and discuss the possible future research associated with these findings.

7.1 Use of *gfp* in ecological studies

This is the first thorough phenotypical comparison between *gfp* transformed entomopathogenic fungi and a wild-type strain, to support the use of *gfp* transformants as substitutes for the wild-type strain in ecological studies. Other studies, using *gfp* transformed entomopathogenic fungi, only compared the virulence of the *gfp* transformants with the wild-type against host arthropods (Inglis *et al*., 2000; Nakazato *et al*., 2006; Cao *et al*., 2007) or provided no comparative details, such as Hu & St. Leger (2002), who stated that the *gfp* transformants of *M. anisopliae* chosen for their field trials had the same growth rate and conidial yield as the parent wild-type; however the protocols for these comparisons were not published. The only detailed comparison between *gfp* transformants and the wild-type strain was by Wu *et al.* (2008), comparing the conidial yield, colony growth and germination rate of *B. bassiana* strain F418 with chosen *gfp* transformants (including *B. bassiana* F418 *gfp* tr1 used in this study). However, only one temperature of incubation was used for the comparison by Wu *et al.* (2008), therefore the current study was more detailed. This study was also the first comparing the persistence of a *gfp* transformed entomopathogenic fungus with the wild-type strain in a soil microcosm, to ensure wild-type and transformed strains behaved similarly, not only *in vitro* but also *in vivo*, as shown in Chapter 3 where *B. bassiana* F418 and F418 *gfp* tr1 had a similar persistence in pasteurised soil with different fertilisers treatments.

The use of a *gfp* transformed fungus with resistance to an antibiotic, such as *B. bassiana* F418 *gfp* tr1 and *gfp* tr3 used in this study (resistant to Hygromycin B), was a useful tool for

studying the population recovery in non-sterile soil, as it allowed specific recovery of *B. bassiana* F418 *gfp* transformants only. *Beauveria bassiana* F418 *gfp* transformants could also be observed in soil suspensions using fluorescent microscopy, however, observation in soil suspensions was challenging due to the low fluorescence emission and the confounding effects of autofluorescence by particles and debris in the soil suspensions. Even though challenging, the use of fluorescence microscopy successfully showed different propagule types (conidia, germinated conidia and piece of mycelia) in soil suspensions in the results presented in Chapter 4. These photographs showed for the first time different propagule types of an entomopathogenic fungi *in vivo* in a soil environment. The use of transformed *B. bassiana* and other entomopathogenic fungi for soil ecology studies could be improved by the production of a transformed strain with a higher level of fluorescence. One possible approach would be the use of synthetic *gfp* cassette (GenScript, USA) based on codon usage preference and using a strong promoter specific to the fungal strain used (Mendoza A., personal communication).

The attempt to locate the insertion point of the *hph-gfp* cassette in the *B. bassiana* F418 *gfp* transformants' genome was also the first report for transformed entomopathogenic fungi. The current study reports the successful isolation of sequences outside the *hph-gfp* cassette. Unfortunately, the lack of full genome sequencing data for *B. bassiana* or close relatives meant that the sequences obtained did not match any known *Beauveria* genes (only matched proteins or putative proteins in different kingdoms). However, more *B. bassiana* sequences are added regularly to databases such as NCBI and it is likely that sequences found in this study will one day match an annotated region in a member of the genus *Beauveria*. A deeper understanding of the link between *gfp* cassette insertion in the fungal genome and the fluorescence level produced is needed and the sequences obtained in this study could be useful to understand that link.

7.2 Factors affecting *Beauveria* in soil

The main objective of Chapter 3 was to understand the effect of some environmental factors in soil on *B. bassiana* F418 persistence in a simplified soil microcosm containing limited microflora (pasteurised soil). The use of a simplified microcosm did help understand the potential impact of each factor (soil moisture, temperature, peat, insects and fertiliser) and reported for the first time the effect of the presence of a non-host arthropod on *B. bassiana* persistence in pasteurised soil. This study showed that *B. bassiana* F418 behaved similarly to other *B. bassiana* strains in response to soil moisture level and soil temperature (Lingg &

Donaldson, 1981; Studdert *et al.*, 1990; Quintela *et al*., 1992). These findings add to the general knowledge of *B. bassiana* survival in soil. However these findings also showed that the impact of individual environmental factors on *B. bassiana* F418 persistence in a simplified microcosm was not reproducible when the complexity of the microcosm microflora increased from pasteurised to non-sterile soil. This was illustrated by the difference observed in *B. bassiana* F418 *gfp* tr1 persistence in pasteurised and non-sterile soil, under different fertiliser amendments, showing that *B. bassiana* F418 *gfp* tr1 populations were affected by the fertilisers used in pasteurised soil but not in non-sterile soil. An overall conclusion of this study was that results from pasteurised or sterile soil were not the same as non-sterile soil and therefore require careful application to real world settings. Although these studies in pasteurised soil have highlighted the likely importance of the soil microflora in the persistence of entomopathogenic fungi in soil, future research on *B. bassiana* should be in non-sterile soil only.

The accidental observation that the temperature at which *B. bassiana* F418 was inoculated in the pasteurised soil seemed to have an impact on population dynamics in the few weeks following the inoculation and even on long term persistence, as observed in Chapter 3, is of interest. No confirmation of this effect could be done in experiments with non-sterile soil in Chapters 4, 5 and 6 as care was taken that the soil temperature during inoculation was the same than subsequent temperature of incubation, however, this finding should be further explored. Faria *et al.* (2009) showed that temperature of imbibition of *B. bassiana* conidia had an impact on their germination rate, which could explain in part the persistence results observed in the present study.

7.3 Impact of soil moisture

Using SSCP approach for microbial community profiling, this study showed a correlation between differences in soil communities and the persistence of *B. bassiana* F418 *gfp* tr3 in pasteurised and non-sterile soil at low and high soil moisture levels. This is the first such study to correlate microbial communities and persistence of *Beauveria*. Lingg & Donaldson (1981), Studdert *et al.* (1990) and Quintela *et al*. (1992) suggested that the difference in *B. bassiana* persistence observed in soil at low and high moisture level was due to a difference in soil microorganisms; however, none could confirm the hypothesis. This study is therefore a step ahead showing the correlation between soil communities and *B. bassiana* F418 *gfp* tr3 persistence in low and high moisture level soils.

Also reported in this thesis was an improvement and successful use of a soil membrane sandwich technique to monitor *B. bassiana* F418 *gfp* tr3 propagules dynamics via quantitative measurement of washed propagules (mostly conidia) and qualitative measurement of mycelium development. Increases in *B. bassiana* populations in soil have been observed (Lingg & Donaldson, 1981; Studdert *et al.*, 1990; Quintela *et al*., 1992) but the propagule type responsible for the increase (conidiation or mycelia development) has never been proven until now. This new technique revealed that *B. bassiana* F418 *gfp* tr3 increase in pasteurised soil was mostly due to mycelia development. In non-sterile soil, *B. bassiana* F418 *gfp* tr3 conidia were probably maintained still for a few months before the dynamics of conidia and mycelia changed with a decrease of conidia and increase in mycelium development in high moisture soil.

However, the use of soil membrane sandwiches had some limitations, such as when using a small membrane pore size, other microorganisms were excluded. This created an unwanted sterile environment. When pores were too big, the fungus could grow beyond the membranes and therefore could not be monitored accurately. A better technique to monitor fungal populations in soil would be the use of q RT PCR based on genes expressed only during germination, mycelium development, conidiation or dormancy. However, finding and monitoring these genes for a specific fungal strain only and optimising the technique would be challenging and was beyond the scope of this PhD project.

New techniques to monitor fungal populations in soil other than soil dilutions plating are needed, and this research has presented an easy and cost effective method, the improved soil membrane sandwich technique, bringing further information on *B. bassiana* F418 *gfp* tr3 population dynamics in soil. This improved technique is an important step and could be used for other entomopathogenic fungi.

The present study showed that should be applied carefully in pastures where *B. bassiana* F418 is applied in order to keep the soil moisture to a level not detrimental to *B. bassiana* F418 survival. Farmers should also be made aware of the risk of loss of *B. bassiana* F418 in soils receiving high rainfall.

7.4 Interactions with non-host insects in soil

This study presented new information on the interaction between an entomopathogenic fungus and a non-host insect. This study showed that persistence of *B. bassiana* F418 and

F418 *gfp* tr3 was better in the pasteurised soil microcosm without insects than in the presence of non-host *C. zealandica* larvae whereas there was no difference between the same treatments in non-sterile soil.

Further investigations showed that *B. bassiana* F418 *gfp* tr3 was able to attach, germinate and form appressoria on the cuticle of live *C. zealandica*. Sitch & Jackson (1997) and Hajek & Eastburn (2003) showed similar findings with *L. lecanii* and *E. maimaiga*, respectively. However, there is no report of a similar finding with *B. bassiana* on a live non-host arthropod, therefore, these results provided new information about *B. bassiana*.

This study showed for the first time a correlation between soil bacterial and fungal communities (using SSCP), particularly bacteria communities, and persistence of *B. bassiana* F418 *gfp* tr3 in pasteurised and non-sterile soil in the presence of non-host insect and without insect.

This study is also the first quantified report of the effect of ingestion of entomopathogenic fungal conidia by a non-host arthropod. The mouth application with a micro-applicator allowed the quantification of conidia ingested by *C. zealandica* larvae and the enumeration of the viable conidia recovered after digestion. Dromph (2001) counted the percentage of viable conidia of *M. anisopliae* after ingestion by collembolans, however, the method used did not allow the quantification of conidia ingested by the collembolans. The method used showed that *C. zealandica* larvae could be able to disperse *B. bassiana* F418 through the field after passage through their guts.

These further investigations led to the conclusion that the decrease of *B. bassiana* F418 *gfp* tr3 in presence of *C. zealandica* in pasteurised soil was either likely due to non-specific germination on non-host cuticle or antagonism by microorganisms associated with *C. zealandica* and the absence of differences in non-sterile soil was probably due to microorganisms inhibiting the non-specific germination on the non-host insect or inhibiting destruction of *B. bassiana* F418 *gfp* tr3 propagules. Further studies would be needed to confirm if only one or both hypotheses affect *B. bassiana* F418 *gfp* tr3 in pasteurised soil, such as testing the microorganisms from *C. zealandica* by retrieving faeces and adding them to the pasteurised soil microcosm. The observation of *C. zealandica* cuticles after sampling the pasteurised soil microcosms did not show any *B. bassiana* F418 *gfp* tr3 conidia attached or germinated, but this could be due to the lower number of conidia in contact with *C. zealandica* cuticles compared to the germination on cuticle experiments. Therefore, further studies using a higher quantity of *B. bassiana* F418 *gfp* tr3 conidia in the pasteurised soil and also a brighter *gfp* transformant could answer the remaining question of the effect of non-specific attachment and germination. The hypothesis that microorganisms in non-sterile

soil inhibits the non-specific germination on *C. zealandica* could be assessed by comparing the germination of *B. bassiana* F418 *gfp* tr3 conidia on non-host cuticle in the presence of filtrate from pasteurised and non-sterile soil. Future research could also include different non-host arthropods to determine if the arthropod species is important for non-host effect.

7.5 Interactions with soil microorganisms

The present study showed the importance of soil microorganisms on *B. bassiana* F418 *gfp* tr3 persistence in soil. When studying the effect of soil moisture level and the presence of no insect/non-host insects in pasteurised and non-sterile soil, a correlation between the changes in soil microbial communities and changes in *B. bassiana* F418 *gfp* tr3 populations was observed. As suggested by Lingg & Donaldson (1981) and Studdert *et al.* (1990), *B. bassiana* persistence in soil is probably not affected by a direct impact of soil moisture level but by an indirect impact of the different microbial communities present in soil at different moisture level. In the present study, we observed a difference in *B. bassiana* F418 *gfp* tr3 populations in pasteurised soil at different moisture levels and in the presence of no insect/non-host insects which correlated to a change in soil microbial communities (mostly bacteria) in both situations. Whereas, in non-sterile soil, *B. bassiana* F418 *gfp* tr3 populations were similar with different moisture levels and in the presence of no insect/non-host insects which correlated to similar soil microbial communities. Unlike the study of Lingg & Donaldson (1981) and Studdert *et al.* (1990) who only hypothesised that soil microorganisms impacted on *B. bassiana* persistence, the present study used SSCP to show a direct correlation between changes in soil microbial communities and *B. bassiana* F418 *gfp* tr3 populations. Although not studied in Chapter 3, it is likely that the difference in *B. bassiana* F418 *gfp* tr1 populations observed in pasteurised soil with different fertiliser was due to a change of microbial communities in the soil.

The presence of *B. bassiana* in non-sterile soil is likely to have a limited impact on other soil microorganisms (Shimazu et al., 2002) as *B. bassiana* is not a good competitor against other microorganisms (Searle & Doberski, 1984). In contrast soil microorganisms can affect *B. bassiana* populations by parasitizing, degrading and/or inhibiting *B. bassiana* (Markova, 1991; Posada *et al*., 2004; Krauss *et al*., 2004; Fargues *et al*., 1983; Lingg & Donaldson, 1981). There is also the well-recognised effect of fungistasis preventing *B. bassiana* germinating in non-sterile soil in the absence of host arthropods (Lockwood, 1964; Clerk, 1969; Walstad *et al*., 1970; Shimazu *et al*., 2002). In the present study, *B. bassiana* F418 *gfp*

tr3 and F418 *gfp* tr1 populations in non-sterile were maintained at the initial inoculum level for a long period of time (monitored up to 3 months) after inoculation in the absence of a host insect. The use of a modified soil sandwich technique showed that *B. bassiana* F418 *gfp* tr3 were mostly maintained as dormant conidia, highlighting the fungistatic effect of microorganisms in non-sterile soil. The fungistatic effect could also help maintaining *B. bassiana* populations at a high level since it prevents germination which can lead to a loss of populations as shown in pasteurised soil with high moisture level. The fungistatic effect might also prevent non-specific germination on non-host insects since *B. bassiana* F418 *gfp* tr3 populations did not decrease in non-sterile soil in the presence of *C. zealandica* larvae, whereas, they decreased in pasteurised soil.

This study shows that soil microbial communities should be studied when investigating the persistence of an entomopathogenic fungus in soil. Soil microbial communities can change with the season (Bardgett *et al.*, 1999), the farming practices such as fertilisation (Bardgett *et al.*, 1999), soil carbon content (Degens *et al*., 2000), soil type (Lupatini *et al*., 2013), climate, pH and soil properties (de Vries *et al*., 2012). The difference in soil microbial communities can affect the degree of fungistasis in soil (Sharapov & Kalvish, 1984; Groden & Lockwood, 1991) and therefore can affect *B. bassiana* persistence and activity in soils.

7.6 *Beauveria* as an endophyte

The experiments carried out in previous chapters were all in bare pasteurised and non-sterile soil; however the interaction between plants and entomopathogenic fungi can be important for persistence and virulence (Hu & St. Leger, 2002; Bruck, 2005; Bing & Lewis, 1991; Akello *et al.*, 2008). Therefore, the last experimental chapter extended the previous chapters to include the effect of plants. New information was generated about the *B. bassiana* F418 by demonstrating the potential as an endophyte in red clover plants. *Beauveria bassiana* F418 *gfp* tr3 could grow as an endophyte in stems and roots of red clover as proven by the culture plating method. However the confirmation using molecular techniques failed, likely due to the high threshold of DNA detection and the low DNA quantity extracted. A better DNA extraction technique should be used for future research and molecular techniques able to detect lower DNA levels should be used such as q-PCR or nested PCR (Enkerli & Widmer, 2010).

The potential ability of *B. bassiana* F418 *gfp* tr3 to be an endophyte in clover plants is of high interest as it could improve the field persistence and could provide targeted control of clover

root weevil if *B. bassiana* F418 can be applied as an endophyte in clover plants. The reduction of pest arthropod by endophytic *B. bassiana* in plants has been shown by Bing & Lewis (1991) and Akello *et al.* (2008) and these studies offer hope that similar findings could be made with *B. bassiana* F418 and the clover plant.

The current study did not show a rhizosphere effect of *B. bassiana* F418 *gfp* tr3 with red clover in non-sterile soil. More studies would be needed to draw conclusions as the length of time of the experiment (18 weeks) and the plant size could have been limiting factors.

7.7 Implications for field applications

This study showed that persistence of *B. bassiana* F418 *gfp* tr3 in pasteurised soil at high moisture level (field capacity) was poor and that a loss of *B. bassiana* F418 *gfp* tr3 conidia stability occurred in non-sterile soil at field capacity (shown using soil the sandwich method). The potential detrimental effect of high soil moisture on *B. bassiana* F418 population in soil could be an issue when *B. bassiana* F418 is applied in pasture where irrigation is used, such as in dairy pasture (Zonderland-Thomassen & Ledgard, 2012), or in regions with high annual rainfall, such as the West Coast, or in soils with high water holding capacity, such as those with high clay or organic matter content. The effect of soil moisture on *B. bassiana* F418 needs to be investigated in field experiments to determine the rate of inoculum loss. This could produce practical information for farmers about the optimal irrigation levels when applying *B. bassiana* F418.

The capacity of *B. bassiana* F418 *gfp* tr3 to attach and germinate on the cuticle of non-host *C. zealandica* larvae *in vitro* is likely to present little issue when applying *B. bassiana* F418 in the field. There was no loss of *B. bassiana* F418 *gfp* tr3 populations in non-sterile soil in the presence of *C. zealandica* larvae, despite the high number of larvae used, which might indicate that fungistasis created by other soil microorganisms prevents non-specific attachment and germination (Walstad *et al*., 1970). The larval stages of *C. zealandica* and *S. lepidus* larvae coincide and are both present in pastures from late autumn to early spring. Thus, the presence of *C. zealandica* larvae may help disperse *B. bassiana* F418 through the pasture as it was shown that *B. bassiana* F418 *gfp* tr3 conidia could be fully recovered and viable after ingestion by *C. zealandica* larvae.

The presence of host *S. lepidus* will likely help maintaining *B. bassiana* F418 populations in pastures as shown previously by Keller *et al*. (2003) and Kessler *et al*. (2004). Although not investigated here, the same mechanism could be true for other non-target hosts, such as

lepidopteran pest Porina (*Wiseana* spp.), which may also contribute to maintaining *B. bassiana* F418 populations at an efficient level for biocontrol (>10^4 spores/g of soil, Nelson *et al*., 2004).

Soil microorganisms were shown to have an important effect on *B. bassiana* F418 *gfp* tr3 populations in this study. The presence of microorganisms antagonistic to *B. bassiana* F418 *gfp* tr3 was the likely explanation for the lower *B. bassiana* F418 *gfp* tr3 populations observed in pasteurised soil. In the pasteurised soil, microorganisms were recolonising empty ecological niches with available nutrients and low competition. The effect was not pronounced in non-sterile soil where microbial communities were shown to be stable. Soil microbial communities change with the season, soil type, soil properties, farming practices and climate (Bardgett *et al.*, 1999; Degens *et al*., 2000; Lupatini *et al*., 2013; de Vries *et al*., 2012). More research is required to thoroughly examine the effect of soil microbial communities in New Zealand pasture soil on *B. bassiana* F418 and this information may improve the use of *B. bassiana* F418 as a biocontrol.

This research showed that there is a potential for application of *B. bassiana* F418 as an endophyte in clover seeds or as a seed coating when sowing pastures. *Beauveria bassiana* has been shown by many researchers to be an endophyte in a broad range of plant species (Bing & Lewis, 1991; Posada & Vega, 2005, 2006; Gomez-Vidal *et al*., 2006; Quesada-Moraga *et al*., 2006; Akello *et al.*, 2007; Tefera & Vidal, 2009 and Gurulingappa *et al.*, 2010). The potential for this biopesticide strain (F418) of *B. bassiana* to be an endophyte in clover plants was demonstrated and this characteristic could be utilised to improve field persistence (even in the absence of hosts) and thereby improve control of clover root weevil (Bing & Lewis, 1991; Akello *et al.*, 2008).

Clover is a standard component of high quality pastures and can be sown either in autumn to renew pastures or spring. *Sitona lepidus* larvae are present in pastures all year round but are more abundant from late autumn to spring where they coincide with establishment of clover pastures. Sowing clover coated with *B. bassiana* F418 in autumn could help controlling the larval population of *S. lepidus* when they first hatch and preventing a high density of *S. lepidus* larvae and adults for the rest of the season. Sowing clover seed coated with *B. bassiana* F418 or as an endophyte inside the seed in spring could help establish the clover plants containing endophytic *B. bassiana* F418. If high moisture level is detrimental to *B. bassiana* F418 persistence in field experiments autumn sowing could present some risk with high rainfalls occurring in autumn and winter.

7.8 Summary

In summary, this study has provided new data on *B. bassiana* persistence in soil including:

- Soil microorganisms have a major impact on the persistence and dynamics of *B. bassiana* F418 and *gfp* transformants.
- The interaction between *B. bassiana* F418 and the non-host *C. zealandica* was shown to be significant for fungal persistence in soil.
- *Beauveria bassiana* F418 *gfp* tr3 can survive endophytically in red clover plants.
- Pasteurised or sterile soil studies do not reflect the effect of factors in non-sterile soil
- *Beauveria bassiana* F418 *gfp* tr1 and tr3 can be used as substitute for the wild-type F418 in ecological studies.
- Membrane sandwiches in soil are a useful technique to monitor *B. bassiana* populations and discriminate qualitatively between conidia and mycelia.
- The use of a micro-applicator to force feed *B. bassiana* F418 *gfp* tr3 conidia to non-host *C. zealandica* larvae is a valuable technique to assess the quantitative effect of ingestion by non-host insect on conidia.

Future research to improve *B. bassiana* F418 as a biocontrol agent should focus on the following points:

- Research using different soil types reflecting New Zealand pasture soils is needed. This could be facilitated by molecular assays that differentiate *B. bassiana* F418 from indigenous strains and do not use genetic engineering of *B. bassiana* so that field experiments are possible.
- Microcosms should include plants to better reflect natural situations. This could be done *in vitro* using the system described in chapter 6. Field experiments would require new molecular tools for strain discrimination to be developed.
- Research should use non-sterile soil only.
- Factors that farmer can manipulate such as pasture plant varietals, fertiliser type and irrigation should be tested more thoroughly.

- Explore the possibility of *B. bassiana* F418 being an endophyte in clover plants and investigate the possibility for the control of clover root weevil.
- New constructs using brighter *gfp* transformed *B. bassiana* F418 are needed for *in vitro* studies.

References

Abbott, W.S. (1925). A method for computing the effectiveness of an insecticide. *J. Econom. Entomol.* 18, 265-7.

Ahmad, J. S., & Baker, R. (1987). Rhizosphere competence of trichoderma-harzianum. *Phytopathology, 77*(2), 182-189.

Akello, J., Dubois, T., Gold, C. S., Coyne, D., Nakavuma, J. & Paparu, P. (2007). *Beauveria bassiana* (Balsamo) Vuillemin as an endophyte in tissue culture banana (*Musa* spp.). *Journal of Invertebrate Pathology, 96*(1), 34-42.

Akello, J., Dubois, T., Coyne, D., & Kyamanywa, S. (2008). Effect of endophytic *Beauveria bassiana* on populations of the banana weevil, *Cosmopolites sordidus*, and their damage in tissue cultured banana plants. *Entomologia Experimentalis et Applicata, 129*(2), 157-165.

Allison, F. R. (1969). A study of the Eugregarines of the grass-grub [larva of *Costelytra zealandica* (White) Melolonthinae], with a description of three new speices. Parasitology, 59, 663-682.

Altre, J. A., & Vandenberg, J. D. (2001). Penetration of cuticle and proliferation in hemolymph by Paecilomyces fumosoroseus isolates that differ in virulence against lepidopteran larvae. *Journal of Invertebrate Pathology, 78*(2), 81-86.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*(3), 403-410.

Bae, Y. & Knudsen, G. R. (2000). Cotransformation of *Trichoderma harzianum* with beta - glucuronidase and green fluorescent protein genes provides a useful tool for monitoring fungal growth and activity in natural soils. *Applied and Environmental Microbiology, 66*(2), 810-815.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., & Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. In *Annual Review of Plant Biology* (Vol. 57, pp. 233-266).

Bardgett, R. D., Lovell, R. D., Hobbs, P. J., & Jarvis, S. C. (1999). Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. *Soil Biology & Biochemistry, 31*(7), 1021-1030.

Barlow, N. D., & Goldson, S. L. (1993). A modeling analysis of the successful biological-control of S*itona discoideus* (Coleoptera: Curculionidae) by *Microctonus aethiopoides* (Hymenoptera: Braconidae) in New Zealand. *Journal of Applied Ecology, 30*(1), 165-178.

Barratt, B. I. P., van Toor, R. F., Ferguson, C. M., Stewart, K. M. (1990). Grass grub and porina in Otago and Southland. A guide to management and control. MAF. 104 pp.

Barratt, B. I. P., Barker, G. M., & Addison, P. J. (1996). *Sitona lepidus* Gyllenhal (Coleoptera: Curculionidae), a potential clover pest new to New Zealand. *New Zealand Entomologist*, 23-30.

Barratt, B. I. P., Evans, A. A., & Ferguson, C. M. (1997). Potential for biocontrol of *Sitona lepidus* Gyllenhal by *Microctonus* spp. *Proceedings of the Fiftieth New Zealand Plant Protection Conference, Lincoln University, Canterbury, New Zealand, 18 21 August, 1997.*

Bassam, B. J., Caetanoanolles, G., & Gresshoff, P. M. (1991). Fast and sensitive silver staining of dna in polyacrylamide gels. *Analytical Biochemistry, 196*(1), 80-83.

Benbouza, H., Jacquemin, J. M., Baudoin, J. P., & Mergeai, G. (2006). Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. *Biotechnologie, Agronomie, Societe et Environnement, 10*(2), 77-81.

Bidochka, M. J. (2001). Monitoring the fate of biocontrol fungi. In *Fungi as Biocontrol Agents: progress, problems and potential* (T. M. Butt, C. W. Jackson & N. Magan, eds): 193-218. CABI Publishing, Wallingford.

Bischoff, J. F., Rehner, S. A. & Humber, R. A. (2009). A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia, 101*(4), 512-530.

Bing, L. A. & Lewis, L. C. (1991). Suppression of *Ostrinia nubilalis* (Hubner) (Lepidoptera, pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. *Environmental Entomology, 20*(4), 1207-1211.

Bittman, S., Forge, T. A. & Kowalenko, C. G. (2005). Responses of the bacterial and fungal biomass in a grassland soil to multi-year applications of dairy manure slurry and fertilizer. *Soil Biology & Biochemistry, 37*(4), 613-623.

Blair, I. D. (1943). Behaviour of the fungus *Rhizoctonia solani* Kuhn in the soil. *Annals of Applied Biology, 30*(2), 118-127.

Boucias, D. (1998). *Insect defense mechanisms - evasion strategies of entomopathogens.* Paper presented at the Proc. 4th International Workshop on Microbial Control of Soil Dwelling Pests, Lincoln, NZ.

Boucias, D. G. & Pendland, J. C. (1998). *Principles of insect pathology*. Kluwer academic publishers, Boston. 548pp.

Boucias, D. G., Pendland, J. C. & Latge, J. P. (1988). Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. *Applied and Environmental Microbiology, 54*(7), 1795-1805.

Brady, B. L. K. (1979). *Beauveria bassiana*. CMI Descriptions of Fungi and Bacteria n°602. Set n° 61. CAB International 1998.

Brownbridge, M., Nelson, T. L., Hackell, D. L., Eden, T. M., Wilson, D. J., Willoughby, B. E. & Glare T.R. (2006). Field application of biopolymer-coated *Beauveria bassiana* F418 for clover root weevil (*Sitona lepidus*) control in Waikato and Manawatu. *New Zealand Plant Protection 59*, 304-311.

Brownbridge, M., Ferguson, C., Saville, D. J., Swaminathan, J., Hurst, M. R. H., & Jackson, T. A. (2008). Potential for biological control of porina (*Wiseana* spp.) with a novel insecticidal bacterium, Yersinia n. sp. (MH96) EN65 strain. *New Zealand Plant Protection, 61*, 229-235.
Bruck, D. J. (2005). Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: implications for pest management. *Biological Control, 32*(1), 155-163.

Cantone, F. A. & Vandenberg, J. D. (1999). Use of the green fluorescent protein for investigations of *Paecilomyces fumosoroseus* in insect hosts. *Journal of Invertebrate Pathology, 74*(2), 193-197.

Cao, Y., Peng, G., He, Z., Wang, Z., Yin, Y. & Xia, Y. (2007). Transformation of *Metarhizium anisopliae* with benomyl resistance and green fluorescent protein genes provides a tag for genetically engineered strains. *Biotechnology Letters, 29*(6), 907-911.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene-expression. *Science, 263*(5148), 802-805.

Chandler, D., Heale, J. B. & Gillespie, A. T. (1993). Competitive interaction between strains of *Verticillium lecanii* on 2 insect hosts. *Annals of Applied Biology, 122*(3), 435-440.

Chase, A. R., Osborne, L. S., & Ferguson, V. M. (1986). Selective isolation of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* from an artificial potting medium. *Florida Entomologist, 69*(2), 285-292.

Christensen, H., Hansen, M. & Sorensen, J. (1999). Counting and size classification of active soil bacteria by fluorescence in situ hybridization with an rRNA oligonucleotide probe. *Applied and Environmental Microbiology, 65*(4), 1753-1761.

Clerk, G. C., & Madelin, M. F. (1965). The longevity of conidia of three insect parasitizing hyphomycetes. *Transactions of the British Mycological Society, 48*(2), 193-209.

Clerk, G. C. (1969). Influence of soil extracts on the germination of conidia of the fungi *Beauveria bassiana* and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology, 13*, 120-124.

Cruz, L. P., Gaitan, A. L. & Gongora, C. E. (2006). Exploiting the genetic diversity of *Beauveria bassiana* for improving the biological control of the coffee berry borer through the use of strain mixtures. *Applied Microbiology and Biotechnology, 71*(6), 918-926.

Degens, B. P., Schipper, L. A., Sparling, G. P., & Vojvodic-Vukovic, M. (2000). Decreases in organic C reserves in soils can reduce the catabolic diversity of soil microbial communities. *Soil Biology & Biochemistry, 32*(2), 189-196.

de Vries, F. T., Hoffland, E., van Eekeren, N., Brussaard, L. & Bloem, J. (2006). Fungal/bacterial ratios in grasslands with contrasting nitrogen management. *Soil Biology & Biochemistry, 38*(8), 2092-2103.

de Vries, F. T., Manning, P., Tallowin, J. R. B., Mortimer, S. R., Pilgrim, E. S., Harrison, K. A., *et al*. (2012). Abiotic drivers and plant traits explain landscape-scale patterns in soil microbial communities. *Ecology Letters, 15*(11), 1230-1239.

Davet, P. & Rouxel, F. (2000). Detection and Isolation of Soil Fungi. Science Publishers, Inc., Enfield, NH, USA; Plymouth, UK.

Doberski, J. W. (1981a). Comparative laboratory studies on 3 fungal pathogens of the elm bark beetle *Scolytus scolytus* - effect of temperature and humidity on infection by *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology, 37*(2), 195-200.

Doberski, J. W. (1981b). Comparative laboratory studies on 3 fungal pathogens of the elm bark beetle *Scolytus scolytus* - pathogenicity of *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus* to larvae and adults of *Scolytus scolytus*. *Journal of Invertebrate Pathology, 37*(2), 188-194.

Dromph, K. M. (2001). Dispersal of entomopathogenic fungi by collembolans. *Soil Biology and Biochemistry, 33*(15), 2047-2051.

Dromph, K. M. & Borgen, A. (2001). Reduction of viability of soil borne inoculum of common bunt (*Tilletia tritici*) by collembolans. *Soil Biology & Biochemistry, 33*(12-13), 1791-1795.

Dromph, K. M. (2003). Collembolans as vectors of entomopathogenic fungi. *Pedobiologia, 47*(3), 245-256.

Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *Bmc Bioinformatics, 5*, 1-19.

Ekesi, S., Maniania, N. K. & Ampong-Nyarko, K. (1999). Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Science and Technology, 9*(2), 177-185.

El-Damir, M. (2006). Variation in germination, virulence and conidial production of single spore isolates of entomopathogenic fungi in response to environmental heterogeneity. *Journal of Biological Sciences, 6*(2), 305-315.

Enkerli, J., & Widmer, F. (2010). Molecular ecology of fungal entomopathogens: molecular genetic tools and their applications in population and fate studies. *BioControl, 55*(1), 17-37.

Fang, W., Leng, B., Xiao, Y., Jin, K., Ma, J., Fan, Y., Feng, J., Yang, X., Zhang, Y & Pei, Y. (2005). Cloning of *Beauveria bassiana* chitinase gene Bbchit1 and its application to improve fungal strain virulence. *Applied and Environmental Microbiology, 71*(1), 363-370.

Fang, W., & Bidochka, M. J. (2006). Expression of genes involved in germination, conidiogenesis and pathogenesis in *Metarhizium anisopliae* using quantitative real-time RT-PCR. *Mycological Research, 110*, 1165-1171.

Fang, W. G. & St. Leger, R. J. (2010). Mrt, a Gene Unique to Fungi, Encodes an Oligosaccharide Transporter and Facilitates Rhizosphere Competency in *Metarhizium robertsii*. *Plant Physiology, 154*(3), 1549-1557.

Fargues, J. F., & Robert, P. H. (1983). Effects of passaging through scarabeid hosts on virulence and host specificity of 2 strains of the entomopathogenic hyphomycete *Metarhizium anisopliae*. *Canadian Journal of Microbiology, 29*(5), 576-583.

Fargues, J., Reisinger, O., Robert, P. H. & Aubart, C. (1983). Biodegradation of entomopathogenic hyphomycetes: influence of clay coating on *Beauveria bassiana* blastospore survival in soil. *Journal of Invertebrate Pathology, 41*(2), 131-142.

Fargues, J., Goettel, M. S., Smits, N., Ouedraogo, A. & Rougier, M. (1997). Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. *Mycologia, 89*(3), 383-392.

Faria, M. R. de, & Wraight, S. P. (2007). Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control, 43*(3), 237-256.

Faria, M., Hajek, A. E., & Wraight, S. P. (2009). Imbibitional damage in conidia of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium acridum*, and *Metarhizium anisopliae*. *Biological Control, 51*(3), 346-354.

Felsenstein, J. (1985). Confidence-limits on phylogenies - an approach using the bootstrap. *Evolution, 39*(4), 783-791.

Feng, M. G. & Johnson, J. B. (1990). Relative virulence of 6 isolates of *Beauveria bassiana* on *Diuraphis noxia* (Homoptera, aphididae). *Environmental Entomology, 19*(3), 785-790.

Feng, M. G., Johnson, J. B. & Kish, L. P. (1990). Virulence of *Verticillium lecanii* and an aphid-derived isolate of *Beauveria bassiana* (fungi, hyphomycetes) for 6 species of cereal-infesting aphids (homoptera, aphididae). *Environmental Entomology, 19*(3), 815-820.

Fernandes, E. K. K., Rangel, D. E. N., Moraes, A. M. L., Bittencourt, V. R. E. P., & Roberts, D. W. (2008). Cold activity of *Beauveria* and *Metarhizium*, and thermotolerance of *Beauveria*. *Journal of Invertebrate Pathology, 98*(1), 69-78.

Fierer, N., Schimel, J. P. & Holden, P. A. (2003). Influence of drying-rewetting frequency on soil bacterial community structure. *Microbial Ecology, 45*(1), 63-71.

Filion, M., St-Arnaud, M., & Jabaji-Hare, S. H. (2003). Direct quantification of fungal DNA from soil substrate using real-time PCR. *Journal of Microbiological Methods, 53*(1), 67-76.

Fisher, J. J., Rehner, S. A., & Bruck, D. J. (2011). Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. *Journal of Invertebrate Pathology, 106*(2), 289-295.

Garraway, M. O. & Evans, R. C. (1984). Fungal Nutrition and Physiology. John Wiley & Sons, Inc., New York, USA.

Gerard, P. J. (2001). Dependence of *Sitona lepidus* (Coleoptera: Curculionidae) larvae on abundance of white clover Rhizobium nodules. *Bulletin of Entomological Research, 91*(2), 149-152.

Gerard, P. J., Goldson, S. L., Hardwick, S., Addison, P. J., & Willoughby, B. E. (2010). The bionomics of an invasive species *Sitona lepidus* during its establishment in New Zealand. *Bulletin of Entomological Research, 100*(3), 339-346.

Gerard, P. J., Wilson, D. J., & Eden, T. M. (2011). Field release, establishment and initial dispersal of Irish *Microctonus aethiopoides* in *Sitona lepidus* populations in northern New Zealand pastures. *BioControl, 56*(6), 861-870.

Gillespie, J. P., Bailey, A. M., Cobb, B. & Vilcinskas, A. (2000). Fungi as elicitors of insect immune responses. *Archives of Insect Biochemistry and Physiology, 44*(2), 49-68.

Glare, T. R., Ocallaghan, M., & Wigley, P. J. (1993). Checklist of naturally-occurring entomopathogenic microbes and nematodes in new-zealand. *New Zealand Journal of Zoology, 20*(2), 95-120.

Goettel, M. S., Eilenberg, J., Glare, T. (2010). Entomopathogenic fungi and their role in regulation of insect populations. In *Insect Control, Biological and Synthetic Agents* (Gilbert, L. I., Gill, S.S.). Academic Press, London, UK.

Gomez-Vidal, S., Lopez-Llorca, L. V., Jansson, H. B. & Salinas, J. (2006). Endophytic colonization of date palm (*Phoenix dactylifera* L.) leaves by entomopathogenic fungi. *Micron, 37*(7), 624-632.

Green, H. & Jensen, D. F. (1995). A Tool for Monitoring *Trichoderma harzianum*: II. The Use of a Gus Transformant for Ecological Studies in the Rhizosphere. *Phytopathology, 85*(11), 1436-1440.

Griffin, D. M. (1963). Soil moisture and the ecology of soil fungi. *Biological reviews of the Cambridge Philosophical Society, 38*(2), 141-166.

Groden, E. & Lockwood, J. L. (1991). Effects of soil fungistasis on *Beauveria bassiana* and its relationship to disease incidence in the Colorado potato beetle, *Leptinotarsa decemlineata*, in Michigan and Rhode Island soils. *Journal of Invertebrate Pathology, 57*(1), 7-16.

Grose, M. J., Parker, C. A, & Sivasithamparam, K. (1984). Growth of *Gaeumannomyces graminis var tritici* in soil - effects of temperature and water potential. *Soil Biology & Biochemistry, 16*(3), 211-216.

Gurulingappa, P., Sword, G. A., Murdoch, G. & McGee, P. A. (2010). Colonization of crop plants by fungal entomopathogens and their effects on two insect pests when in planta. *Biological Control, 55*(1), 34-41.

Hackell, D. L. & Gerard, P. J. (2004). Nodule preference by first instar clover root weevil. *New Zealand Plant Protection, Volume 57, 2004 Proceedings of a conference, Hamilton, New Zealand, 10 12 August 2004.*

Hajek, A. E. & Eastburn, C. C. (2003). Attachment and germination of *Entomophaga maimaiga* conidia on host and non-host larval cuticle. *Journal of Invertebrate Pathology, 82*(1), 12-22.

Hallsworth, J. E., & Magan, N. (1999). Water and temperature relations of growth of the entomogenous fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology, 74*(3), 261-266.

Hardwick, S. (1998). Laboratory investigations into feeding preferences of adult *Sitona lepidus* Gyllenhal. *Proceedings of the Fifty First New Zealand Plant Protection Conference, Quality Hotel, Hamilton, New Zealand, 11-13 August, 1998*, 5-8.

Harris, J. N., New, P. B., & Martin, P. M. (2006). Laboratory tests can predict beneficial effects of phosphate-solubilising bacteria on plants. *Soil Biology & Biochemistry, 38*(7), 1521-1526.

Harrison, R. D., & Gardner, W. A. (1991). Occurrence of the entomogenous fungus *Beauveria bassiana* in pecan orchard soils in Georgia. *Journal of Entomological Science, 26*(3), 360-366.

Holder, D. J. & Keyhani, N. O. (2005). Adhesion of the entomopathogenic fungus *Beauveria* (Cordyceps) *bassiana* to substrata. *Applied and Environmental Microbiology, 71*(9), 5260-5266.

Hohmann, P. (2010). Understanding *Trichoderma* bio-inoculants in the root ecosystem of *Pinus radiata* seedlings. PhD thesis. Lincoln University, New Zealand.

Hong, T. D., Gunn, J., Ellis, R. H., Jenkins, N. E. & Moore, D. (2001). The effect of storage environment on the longevity of conidia of *Beauveria bassiana*. *Mycological Research, 105*, 597-602.

Hori, T., Haruta, S., Ueno, Y., Ishii, M., & Igarashi, Y. (2006). Direct comparison of single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) to characterize a microbial community on the basis of 16S rRNA gene fragments. *Journal of Microbiological Methods, 66*(1), 165-169.

Hu, G. & St. Leger, R. J. (2002). Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Applied and Environmental Microbiology, 68*(12), 6383-6387.

Huber, J. (1958). Untersuchungen zur Physiologie insektentötender Pilze. *Arch. Mikrobiol*., **29**, 257-276.

Hughes, W. O. H., Thomsen, L., Eilenberg, J., & Boomsma, J. J. (2004). Diversity of entomopathogenic fungi near leaf-cutting ant nests in a neotropical forest, with particular reference to *Metarhizium anisopliae* var. *anisopliae*. *Journal of Invertebrate Pathology, 85*(1), 46-53.

Hywel-Jones, N. L. & Gillespie, A. T. (1990). Effect of temperature on spore germination in *Metarhizium anisopliae* and *Beauveria bassiana*. *Mycological Research, 94*, 389-392.

Inglis, G. D., Ivie, T. J., Duke, G. M. & Goettel, M. S. (2000). Influence of rain and conidial formulation on persistence of *Beauveria bassiana* on potato leaves and Colorado potato beetle larvae. *Biological Control, 18*(1), 55-64.

Inglis, G. D., Goettel, M. S., Butt, T. M. & Strasser, H. (2001). Use of hyphomycetous fungi for managing insect pests. In *Fungi as Biocontrol Agents: progress, problems and potential* (T. M. Butt, C. W. Jackson & N. Magan, eds): 23-70. CABI Publishing, Wallingford.

Jackson, T. A., Huger, A. M. & Glare, T. R. (1993). Pathology of amber disease in the New Zealand grass grub *Costelytra zealandica* (Coleoptera, scarabaeidae). *Journal of Invertebrate Pathology, 61*(2), 123-130.

Jackson, D., Zemenick, K., & Huerta, G. (2012). Occurrence in the soil and dispersal of *Lecanicillium lecanii*, a fungal pathogen of the green coffee scale (*Coccus viridis*) and coffee rust (*Hemileia vastatrix*). *Tropical and Subtropical Agroecosystems, 15*(2), 389-401.

James, R.R. (2001). Effects of exogenous nutrients on conidial germination and virulence against the silverleaf whitefly for two hyphomycetes. *Journal of invertebrate Pathology*, *77*(2), 99-107.

Jaronski, S. T. (2007). Soil ecology of the entomopathogenic Ascomycetes: a critical examination of what we (think) we know. In *Use of Entomopathogenic Fungi in Biological Pest Management* (Ekesi, S. & Maniania, N.K. eds): 91-144. Research signpost, Kerala, India.

Jingguo, W. & Bakken, L. R. (1997). Competition for nitrogen during mineralization of plant residues in soil: Microbial response to C and N availability. *Soil Biology & Biochemistry, 29*(2), 163-170.

Jones, W. E., Grace, J. K. & Tamashiro, M. (1996). Virulence of seven isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to *Coptotermes formosanus* (Isoptera: Rhinotermitidae). *Environmental Entomology, 25*(2), 481-487.

Katznelson, H. (1940). Survival of microorganisms inoculated into sterilized soil. *American Association for the Advancement of Science. Science, 49*, 211-217.

Keller, S., Kessler, P., & Schweizer, C. (2003). Distribution of insect pathogenic soil fungi in Switzerland with special reference to *Beauveria brongniartii* and *Metharhizium anisopliae*. *BioControl, 48*(3), 307-319.

Kessler, P., & Keller, S. (2003). Influence of soil environment on growth and persistence of Beauveria brongniartii. *Bulletin OILB/SROP, 26*(1), 99-102.

Kessler, P., Matzke, H., & Keller, S. (2003). The effect of application time and soil factors on the occurrence of *Beauveria brongniartii* applied as a biological control agent in soil. *Journal of Invertebrate Pathology, 84*(1), 15-23.

Kessler, P., Enkerli, J., Schweizer, C. & Keller, S. (2004). Survival of *Beauveria brongniartii* in the soil after application as a biocontrol agent against the European cockchafer *Melolontha melolontha*. *BioControl, 49*(5), 563-581.

Knudsen, G. R. & Bin, L. (1990). Effects of temperature, soil moisture, and wheat bran on growth of *Trichoderma harzianum* from alginate pellets. *Phytopathology, 80*(8), 724-727.

Krauss, U., Hidalgo, E., Arroyo, C., & Piper, S. R. (2004). Interaction between the entomopathogens *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* and the mycoparasites *Clonostachys* spp., *Trichoderma harzianum* and *Lecanicillium lecanii*. *Biocontrol Science and Technology, 14*(4), 331-346.

Krueger, S. R., Nechols, J. R., & Ramoska, W. A. (1991). Infection of chinch bug, *Blissus leucopterusleucopterus* (Hemiptera: Lygaeidae), adults from *Beauveria bassiana* (Deuteromycotina, Hyphomycetes) conidia in soil under controlled temperature and moisture conditions. *Journal of Invertebrate Pathology, 58*(1), 19-26.

Krueger, S. R., & Roberts, D. W. (1997). Soil treatment with entomopathogenic fungi for corn rootworm (*Diabrotica* spp.) larval control. *Biological Control, 9*(1), 67-74.

Kullman, B., Tamm, H. & Kullman, K. (2005). Fungal genome size database. *http://www.zbi.ee/fungal-genomesize.*

Lacey, C. M., Lacey, L. A. & Roberts, D. R. (1988). Route of invasion and histopathology of metarhizium-anisopliae in culex-quinquefasciatus. *Journal of Invertebrate Pathology, 52*(1), 108-118.

Li, Z. Z., Li, C. R., Huang, B., & Fan, M. Z. (2001). Discovery-and demonstration of the teleomorph of Beauveria bassiana (Bals.) Vuill., an important entomogenous fungus. *Chinese Science Bulletin, 46*(9), 751-753.

Li, Z. Z. (1988). List on the insect hosts of *Beauveria bassiana*. In: *Study and application of entomogenous fungi in China*, Vol. 1. Beijing: Academic Periodical Press. pp 241_255 (in Chinese with Latin names and English summary).

Lingg, A. J. & Donaldson, M. D. (1981). Biotic and abiotic factors affecting stability of *Beauveria bassiana* conidia in soil. *Journal of Invertebrate Pathology, 38*(2), 191-200.

Liu, Y. G. & Huang, N. (1998). Efficient amplification of insert end sequences from bacterial artificial chromosome clones by thermal asymmetric interlaced PCR. *Plant Molecular Biology Reporter, 16*(2), 175-181.

Lockwood, J. L. (1964). Soil fungistasis. *Ann. Rev. Phytopath., 2*, 341-362.

Lorang, J. M., Tuori, R. P., Martinez, J. P., Sawyer, T. L., Redman, R. S., Rollins, J. A., Wolpert, T.J., Johnson, K.B., Rodriguez, R.J., Dickman, M.B. & Ciufetti, L.M. (2001). Green fluorescent protein is lighting up fungal biology. *Applied and Environmental Microbiology, 67*(5), 1987-1994.

Lord, J. C. (2005). From Metchnikoff to Monsanto and beyond: the path of microbial control. *Journal of Invertebrate Pathology, 89*(1), 19-29.

Lotan, R., Berdicevsky, I., Merzbach, D., & Grossowicz, N. (1976). Effect of calcium ions on growth and metabolism of *Saccharomyces carlsbergensis*. *Journal of general microbiology, 92*(1), 76-80.

Lupatini, M., Jacques, R. J. S., Antoniolli, Z. I., Suleiman, A. K. A., Fulthorpe, R. R., & Roesch, L. F. W. (2013). Land-use change and soil type are drivers of fungal and archaeal communities in the Pampa biome. *World journal of microbiology & biotechnology, 29*(2), 223-233.

Luz, C. & Fargues, J. (1997). Temperature and moisture requirements for conidial germination of an isolate of *Beauveria bassiana*, pathogenic to *Rhodnius prolixus*. *Mycopathologia, 138*(3), 117-125.

Luz, C. & Fargues, J. (1999). Dependence of the entomopathogenic fungus, *Beauveria bassiana*, on high humidity for infection of *Rhodnius prolixus*. *Mycopathologia, 146*(1), 33-41.

Majchrowicz, I., Poprawski, T. J., Robert, P. H. & Maniania, N. K. (1990). Effects of entomopathogenic and opportunistic fungi on *Delia antiqua* (diptera, anthomyiidae) at low relative-humidity. *Environmental Entomology, 19*(4), 1163-1167.

Mander, C. (2006). Investigation of the pathogenicity and persistence of *Beauveria bassiana* for biological control of Fuller's rose weevil larvae in soil. PhD thesis. Lincoln University, New Zealand.

Markova, G. (1991). *Melanospora parasitica* attacking *Beauveria bassiana* on ash weevil *Stereonychus fraxini*, in Bulgaria. *Journal of Invertebrate Pathology, 58*(3), 450-452.

Marschner, P. & Rumberger, A. (2004). Rapid changes in the rhizosphere bacterial community structure during re-colonization of sterilized soil. *Biology and Fertility of Soils, 40*(1), 1-6.

Mckinnon, A. (2011). Rhizosphere colonisation of *Beauveria* Vuillemin species (Ascomycota: Hypocreales) (*B. bassiana* and *B. caledonica*). Master thesis. Lincoln University, New Zealand.

Mekwatanakarn, P. & Sivasithamparam, K. (1987). Effect of certain herbicides on soil microbial-populations and their influence on saprophytic growth in soil and pathogenicity of take-all fungus. *Biology and Fertility of Soils, 5*(2), 175-180.

Ment, D., Churchill, A. C. L., Gindin, G., Belausov, E., Glazer, I., Rehner, S. A., et al. (2012). Resistant ticks inhibit *Metarhizium* infection prior to haemocoel invasion by reducing fungal viability on the cuticle surface. *Environmental microbiology, 14*(6), 1570-1583.

Meyling, N. V., & Eilenberg, J. (2006). Isolation and characterisation of *Beauveria bassiana* isolates from phylloplanes of hedgerow vegetation. *Mycological Research, 110*, 188-195.

Meyling, N. V., Lubeck, M., Buckley, E. P., Eilenberg, J., & Rehner, S. A. (2009). Community composition, host range and genetic structure of the fungal entomopathogen Beauveria in adjoining agricultural and seminatural habitats. *Molecular Ecology, 18*(6), 1282-1293.

Meyling, N. V., Thorup-Kristensen, K., & Eilenberg, J. (2011). Below- and aboveground abundance and distribution of fungal entomopathogens in experimental conventional and organic cropping. *Biological Control, 59*(2), 180-186.

Meyling, N. V., Schmidt, N. M., & Eilenberg, J. (2012). Occurrence and diversity of fungal entomopathogens in soils of low and high Arctic Greenland. *Polar Biology, 35*(9), 1439-1445.

Nakazato, L., Dutra, V., Broetto, L., Staats, C. C., Vainstein, M. H., & Schrank, A. (2006). Development of an expression vector for *Metarhizium anisopliae* based on the tef-1 alpha homologous promoter. *Applied Microbiology and Biotechnology, 72*(3), 521-528.

Nelson, T. L., Willoughby, B. E., Wilson, D. J., Eden, T. & Glare, T. R. (2004). Establishing the fungus *Beauveria bassiana* in pasture for clover root weevil (*Sitona lepidus*) control. *New Zealand Plant Protection, 57*, 314-318.

New Zealand Soil Bureau (1968). Templeton Silt Loam Pedology. In *Soils of New Zealand, Part 3*, Soil Bureau Bulletin 26 (3). New Zealand Department of Scientific and Industrial Research, Wellington. 127 p.

O'Brien, T. (2009). The saprophytic life of *Metarhizium anisopliae*. PhD thesis. University of Maryland, College Park, USA.

O'-Callaghan, M., Gerard, E. M. & Johnson, V. W. (2001). Effect of soil moisture and temperature on survival of microbial control agents. *New Zealand Plant Protection*, *54*, 128-135.

Olsen, C. H. (2003). Review of the Use of Statistics in Infection & Immunity. Infection & Immunity 71, 6689-6692.

Orr, K. A., & Knudsen, G. R. (2004). Use of green fluorescent protein and image analysis to quantify proliferation of *Trichoderma harzianum* in nonsterile soil. *Phytopathology, 94*(12), 1383-1389.

Ownley, B. H., Bishop, D. G., Pereira, R. M. (2000). Biocontrol of *Rhizoctonia* damping-off of tomato with Beauveria bassiana. *Phytopathology*, 90:S58.

Ownley, B. H., Pereira, R. M., Klingeman, W. E., Quigley, N. B., Leckie, B. M. (2004). *Beauveria bassiana*, a dual purpose biocontrol organism, with activity against insect pests and plant pathogens. In *Emerging concepts in plant health management* (Lartey, R. T., Caesar, A., eds): 256-269. Research Signpost, Kerala.

Ownley, B. H., Gwinn, K. D., & Vega, F. E. (2010). Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. *BioControl, 55*(1), 113-128.

Pathan, A. A. K., Devi, K. U., Vogel, H., & Reineke, A. (2007). Analysis of differential gene expression in the generalist entomopatho genic fungus *Beauveria bassiana* (Bals.) Vuillemin grown on different insect cuticular extracts and synthetic medium through cDNA-AFLPs. *Fungal Genetics and Biology, 44*(12), 1231-1241.

Paulitz, T. C. (2000). Population dynamics of biocontrol agents and pathogens in soils and rhizospheres. *European Journal of Plant Pathology, 106*(5), 401-413.

Pereira, R. M., Alves, S. B., & Stimac, J. L. (1993). Growth of *Beauveria bassiana* in fire ant nest soil with amendments. *Journal of Invertebrate Pathology, 62*(1), 9-14.

Phillips, C. B., Goldson, S. L., Reimer, L., & Kuhlmann, U. (2000). Progress in the search for biological control agents of clover root weevil, *Sitona lepidus* (Coleoptera: Curculionidae). *New Zealand Journal of Agricultural Research, 43*(4), 541-547.

Phillips, C. B., McNeill, M. R., Hardwick, S., Vink, C. J., Kean, J. M., Bewsell, D., et al. (2007). Clover root weevil in the South Island: detection, response and current distribution. *New Zealand Plant Protection, 60*, 209-216.

Phillips, C. B., McNeill, M. R., Hardwick, S., Ferguson, C. M., & Kean, J. M. (2010). South Island distribution of clover root weevil and its biocontrol agent. *Proceedings of the New Zealand Grassland Association, 72*, 227-232.

Posada, F., Vega, F. E., Rehner, S. A., Blackwell, M., Weber, D., Suh, S. O., et al. (2004). *Syspastospora parasitica*, a mycoparasite of the fungus *Beauveria bassiana* attacking the Colorado potato beetle *Leptinotarsa decemlineata*: A tritrophic association. *Journal of Insect Science, 4*.

Posada, F. & Vega, F. E. (2005). Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in cocoa seedlings (*Theobroma cacao*). *Mycologia, 97*(6), 1195-1200.

Posada, F. & Vega, F. E. (2006). Inoculation and colonization of coffee seedlings (*Coffea arabica* L.) with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). *Mycoscience, 47*(5), 284-289.

Quesada-Moraga, E., Landa, B. B., Munoz-Ledesma, J., Jimenez-Diaz, R. M. & Santiago-Alvarez, C. (2006). Endophytic colonisation of opium poppy, *Papaver somniferum*, by an entomopathogenic *Beauveria bassiana* strain. *Mycopathologia, 161*(5), 323-329.

Quintela, E. D., Lord, J. C., Alves, S. B. & Roberts, D. W. (1992). Persistence of *Beauveria bassiana* in cerrado soil and its interaction with soil microorganisms. *Anais da Sociedade Entomologica do Brasil, 21*(1), 69-82.

Raveh, A. & Avnimelech, Y. (1978). The effect of drying on the colloidal properties and stability of humic compounds. *Plant and Soil, 50*(3), 545-552.

Rath, A. C., Koen, T. B., & Yip, H. Y. (1992). The influence of abiotic factors on the distribution and abundance of *Metarhizium anisopliae* in Tasmanian pasture soils. *Mycological Research, 96*, 378-384.

Reay, S. D., Brownbridge, M., Gicquel, B., Cummings, N. J. & Nelson, T. L. (2010). Isolation and characterization of endophytic *Beauveria* spp. (Ascomycota: Hypocreales) from *Pinus radiata* in New Zealand forests. *Biological Control, 54*(1), 52-60.

Rehner, S. A. & Buckley, E. (2005). A *Beauveria* phyloggeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia, 97*(1), 84-98.

Rehner, S. A., Minnis, A. M., Sung, G.-H., Luangsa-ard, J. J., Devotto, L. & Humber, R. A. (2011). Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. *Mycologia, 103*(5), 1055-1073.

Rodríguez-Lozano, A., Campagnoli, M., Jewel, K., Monadjemi, F. & Gaze, J. E. (2010). *Bacillus* spp. thermal resistance and validation in soup. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. A. Méndez-Vilas (Ed.)Formatex.

Rosin, F., Shapiro, D. I. & Lewis, L. C. (1996). Effects of fertilizers on the survival of *Beauveria bassiana*. *Journal of Invertebrate Pathology, 68*(2), 194-195.

Rousk, J., Baath, E., Brookes, P. C., Lauber, C. L., Lozupone, C., Caporaso, J. G., Knight, R. & Fierer N. (2010). Soil bacterial and fungal communities across a pH gradient in an arable soil. *Isme Journal, 4*(10), 1340-1351.

Safavi, S. A., Shah, F. A., Pakdel, A. K., Rasoulian, G. R., Bandani, A. R., & Butt, T. M. (2007). Effect of nutrition on growth and virulence of the entomopathogenic fungus *Beauveria bassiana*. *Fems Microbiology Letters, 270*(1), 116-123.

Saitou, N. & Nei, M. (1987). The neighbor-joining method - a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406-425.

Sandhu, S. S., Rajak, R. C. & Agarwal, G. P. (1993). Studies on prolonged storage of *Beauveria bassiana* conidia - effects of temperature and relative-humidity on conidial viability and virulence against chickpea borer, *Helicoverpa armigera*. *Biocontrol Science and Technology, 3*(1), 47-53.

Sanguinetti, C. J., Neto, E. D. & Simpson, A. J. G. (1994). Rapid silver staining and recovery of pcr products separated on polyacrylamide gels. *Biotechniques, 17*(5), 914-&.

Sarathchandra, S. U., Ghani, A., Yeates, G. W., Burch, G. & Cox, N. R. (2001). Effect of nitrogen and phosphate fertilisers on microbial and nematode diversity in pasture soils. *Soil Biology & Biochemistry, 33*(7-8), 953-964.

Sasan, R. K., & Bidochka, M. J. (2012). The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *American Journal of Botany, 99*(1), 101-107.

Schabel, H. G. (1976). Oral infection of *Hylobius pales* by *Metarrhizium anisopliae*. *Journal of Invertebrate Pathology, 27*(3), 377-383.

Scheepmaker, J. W. A., & Butt, T. M. (2010). Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment and in accordance with EU regulations. *Biocontrol Science and Technology, 20*(5), 503-552.

Schmidt, E. L. (1979). Initiation of plant root-microbe interactions. *Annual review of microbiology, 33*, 355-376.

Schwieger, F. & Tebbe, C. C. (1998). A new approach to utilize PCR-single-strand-conformation polymorphism for 16s rRNA gene-based microbial community analysis. *Applied and Environmental Microbiology, 64*(12), 4870-4876.

Searle, T., & Doberski, J. (1984). An investigation of the entomogenous fungus *Beauveria bassiana* (bals) vuill as a potential biological-control agent for *Oryzaephilus surinamensis* (l). *Journal of Stored Products Research, 20*(1), 17-23.

Shah, F. A., Wang, C. S. & Butt, T. M. (2005). Nutrition influences growth and virulence of the insect-pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiology Letters, 251*(2), 259-266.

Sharapov, V. M. & Kalvish, T. K. (1984). Effect of soil fungistasis on zoopathogenic fungi. *Mycopathologia, 85*(1-2), 121-128.

Shimazu, M., Maehara, N., & Sato, H. (2002). Density dynamics of the entomopathogenic fungus, *Beauveria bassiana* Vuillemin (Deuteromycotina: Hyphomycetes) introduced into forest soil, and its influence on other soil microorganisms. *Applied Entomology and Zoology, 37*(2), 263-269.

Simon, A., Sivasithamparam, K. & Macnish, G. C. (1988). Effect of application to soil of nitrogenous fertilizers and lime on biological suppression of *Gaeumannomyces graminis* var *tritici*. *Transactions of the British Mycological Society, 91*, 287-294.

Sitch, J. C. & Jackson, C. W. (1997). Pre-penetration events affecting host specificity of *Verticillium lecanii*. *Mycological Research, 101*, 535-541.

Sivan, A., & Harman, G. E. (1991). Improved rhizosphere competence in a protoplast fusion progeny of *Trichoderma harzianum*. *Journal of general microbiology, 137*, 23-29.

Smith, R. J. & Grula, E. A. (1981). Nutritional-requirements for conidial germination and hyphal growth of *Beauveria bassiana*. *Journal of Invertebrate Pathology, 37*(3), 222-230.

Smith, R. J., Pekrul, S., & Grula, E. A. (1981). Requirement for sequential enzymatic-activities for penetration of the integument of the corn-earworm (*Heliothis zea*). *Journal of Invertebrate Pathology, 38*(3), 335-344.

Spellig, T., Bottin, A. & Kahmann, R. (1996). Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. *Molecular & General Genetics, 252*(5), 503-509.

St. Leger, R. J., Frank, D. C., Roberts, D. W., & Staples, R. C. (1992). Molecular cloning and regulatory analysis of the cuticle-degrading-protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. *European Journal of Biochemistry, 204*(3), 991-1001.

Stoffella, P. J., & Fleming, M. F. (1990). Plant-population influences yield variability of cabbage. *Journal of the American Society for Horticultural Science, 115*(5), 708-711.

Strasser, H., Forer, A. & Schinner, F. (1996). Development of media for the selective isolation and maintenance of virulence of *Beauveria brongniartii*. In *Microbial Control of Soil Dwelling Pests* (T. A. Jackson & T. R. Glare, eds): 125-130. AgResearch, Lincoln, NZ.

Studdert, J. P., Kaya, H. K. & Duniway, J. M. (1990). Effect of water potential, temperature, and clay-coating on survival of *Beauveria bassiana* conidia in a loam and peat soil. *Journal of Invertebrate Pathology, 55*(3), 417-427.

Studdert, J. P., & Kaya, H. K. (1990). Water potential, temperature, and clay-coating of *Beauveria bassiana* conidia - effect on *Spodoptera exigua* pupal mortality in 2 soil types. *Journal of Invertebrate Pathology, 56*(3), 327-336.

Sturz, A. V., Christie, B. R., Matheson, B. G., & Nowak, J. (1997). Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils, 25*(1), 13-19.

Takai, K., & Horikoshi, K. (2000). Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Applied and Environmental Microbiology, 66*(11), 5066.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution, 28*(10), 2731-2739.

Tartar, A. & Boucias, D. G. (2004). A pilot-scale Expressed Sequence Tag analysis of *Beauveria bassiana* gene expression reveals a tripeptidyl peptidase that is differentially expressed in vivo. *Mycopathologia, 158*(2), 201-209.

Tefera, T. & Vidal, S. (2009). Effect of inoculation method and plant growth medium on endophytic colonization of sorghum by the entomopathogenic fungus *Beauveria bassiana*. *BioControl, 54*(5), 663-669.

Thomas, M. B., & Read, A. F. (2007). Opinion - Can fungal biopesticides control malaria? *Nature Reviews Microbiology, 5*(5), 377-383.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). Clustal-w - improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*(22), 4673-4680.

Thornton, C. R. (2004). An immunological approach to quantifying the saprotrophic growth dynamics of *Trichoderma* species during antagonistic interactions with *Rhizoctonia solani* in a soil-less mix. *Environmental microbiology, 6*(4), 323-334.

Townsend, R. J., O'-Callaghan, M., Johnson, V. W., & Jackson, T. A. (2003). Compatibility of microbial control agents *Serratia entomophila* and *Beauveria bassiana* with selected fertilisers. *New Zealand Plant Protection, Volume 56, 2003 Proceedings of a conference, Chateau on the Park, Christchurch, New Zealand, 12 14 August 2003.*

Treseder, K. K. (2008). Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. *Ecology Letters, 11*(10), 1111-1120.

Vanden Wymelenberg, A. J., Cullen, D., Spear, R. N., Schoenike, B. & Andrews, J. H. (1997). Expression of green fluorescent protein in *Aureobasidium pullulans* and quantification of the fungus on leaf surfaces. *Biotechniques, 23*(4), 686-690.

Vänninen, I. (1996). Distribution and occurrence of four entomopathogenic fungi in Finland: Effect of geographical location, habitat type and soil type. *Mycological Research, 100*, 93-101.

Vänninen, I., Tyni-Juslin, J., & Hokkanen, H. (2000). Persistence of augmented *Metarhizium anisopliae* and *Beauveria bassiana* in Finnish agricultural soils. *BioControl, 45*(2), 201-222.

Vey, A., Hoagland, R. E. & Butt, T. M. (2001). Toxic metabolites of fungal biocontrol agents. In *Fungi as Biocontrol Agents: progress, problems and potential* (T. M. Butt, C. W. Jackson & N. Magan, eds): 311-346. CABI Publishing, Wallingford.

Viaud, M., Couteaudier, Y., Levis, C., & Riba, G. (1996). Genome organization in *Beauveria bassiana*: Electrophoretic karyotype, gene mapping, and telomeric fingerprint. *Fungal Genetics and Biology, 20*(3), 175-183.

Vilcinskas, A. & Gotz, P. (1999). Parasitic fungi and their interactions with the insect immune system. *Advances in Parasitology, 43*, 267-313.

Wagner, B. L. & Lewis, L. C. (2000). Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. *Applied and Environmental Microbiology, 66*(8), 3468-3473.

Wakelin, S. A., Sivasithamparam, K., Cole, A. L. J. & Skipp, R. A. (1999). Saprophytic growth in soil of a strain of *Trichoderma koningii*. *New Zealand Journal of Agricultural Research, 42*(3), 337-345.

Walker, T. S., Bais, H. P., Grotewold, E., & Vivanco, J. M. (2003). Root exudation and rhizosphere biology. *Plant Physiology, 132*(1), 44-51.

Walstad, J. D., Anderson, R. F. & Stambaugh, W. J. (1970). Effects of environmental conditions on two species of muscardine fungi (*Beauveria bassiana* and *Metarrhizium anisopliae*). *Journal of Invertebrate Pathology, 16*(2), 221-226.

Wang, S., O'Brien, T. R., Pava-Ripoll, M., & St Leger, R. J. (2011). Local adaptation of an introduced transgenic insect fungal pathogen due to new beneficial mutations. *Proceedings of the National Academy of Sciences of the United States of America, 108*(51), 20449-20454.

Wang, L., Huang, J., You, M. & Liu, B. (2004). Time-dose-mortality modelling and virulence indices for six strains of *Verticillium lecanii* against sweet potato whitefly, *Bemisia tabaci* (Gennadius). *Journal of Applied Entomology, 128*(7), 494-500.

Wang, C., & St. Leger, R. J. (2007). The *Metarhizium anisopliae* perilipin homolog MPL1 regulates lipid metabolism, appressorial turgor pressure, and virulence. *Journal of Biological Chemistry, 282*(29), 21110-21115.

Warcup, J. H. (1955). On the origin of colonies of fungi developing on soil dilution plates. *Transactions of the British Mycological Society, 38*, 298-230.

Watanabe, K., Kodama, Y. & Harayama, S. (2001). Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. *Journal of Microbiological Methods, 44*(3), 253-262.

Watson, R. N., Eerens, J. P. J., & Davis, L. T. (2002). Clover root weevil feeding and larval numbers on white and red clover seedlines. *New Zealand Plant Protection*, 55, 252-257.

Wear, S., Andrews, G. (2005). Clover root weevil economic impact assessment: Report to Biosecurity New Zealand, Ministry of Agriculture and Forestry, Wellington. New Zealand Institute of Economic Research, wellington, New Zealand. 35 pp.

Willoughby, B. E., Glare, T. R., Kettlewell, F. J., & Nelson, T. L. (1998). *Beauveria bassiana* as a potential biocontrol agent against the clover root weevil, *Sitona lepidus*. *Proceedings of the Fifty First New Zealand Plant Protection Conference, Quality Hotel, Hamilton, New Zealand, 11 13 August, 1998.*

White, T.M., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA, pp. 315–321.

Wraight, S. P., Jackson, M. A. & de Kock, S. L. (2001). Production, stabilization and formulation of fungal biocontrol agents. In *Fungi as Biocontrol Agents: progress, problems and potential* (T. M. Butt, C. W. Jackson & N. Magan, eds): 253-288. CABI Publishing, Wallingford.

Wu, J., Ridgway, H., Carpenter, M. & Glare, T. (2008). Efficient transformation of *Beauveria bassiana* by *Agrobacterium tumefaciens*-mediated insertional mutagenesis. *Australasian Plant Pathology, 37*(6), 537-542.

Wu, Y., He, Y., Wang, H. Xu, J. (2010). Effects of Soil Water Content on Soil Microbial Biomass and Community Structure Based on Phospholipid Fatty Acid Analysis. In Molecular Environment Soil Science at the Interface in the Earth's Critical Zone.

Wyrebek, M., Huber, C., Sasan, R. K., & Bidochka, M. J. (2011). Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. *Microbiology-Sgm, 157*, 2904-2911.

Zhang, H., & Jackson, T. A. (2008). Autochthonous bacterial flora indicated by PCR-DGGE of 16S rRNA gene fragments from the alimentary tract of *Costelytra zealandica* (Coleoptera: Scarabaeidae). *Journal of Applied Microbiology, 105*(5), 1277-1285.

Zimmermann, G. (1986). The *Galleria* bait method for detection of entomopathogenic fungi in soil. *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie, 102*(2), 213-215.

Zimmermann, G., Papierok, B., & Glare, T. (1995). Elias Metschnikoff, Elie Metchnikoff or Ilya Ilich Mechnikov (1845-1916): A pioneer in insect pathology, the first describer of the entomopathogenic fungus *Metarhizium anisopliae* and how to translate a Russian name. *Biocontrol Science and Technology, 5*(4), 527-530.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology, 17*(5-6), 553-596.

Zonderland-Thomassen, M. A., & Ledgard, S. F. (2012). Water footprinting - A comparison of methods using New Zealand dairy farming as a case study. *Agricultural Systems, 110*, 30-40.

Presentations from thesis

2010 **Blond, C.**, Glare, T., Ridgway, H., Chapman, R., Condron, L. and Brownbridge, M. (2010). Effects of the presence of susceptible and non-susceptible insects on *Beauveria bassiana* F418 *gfp* tr3 persistence in soil. Oral presentation at the 43rd Annual Meeting of the Society for Invertebrate Pathology, 11-15 July 2010, Trabzon, Turkey.

2009 **Blond, C**., Ridgway, H., Glare, T., Condron, L. and Brownbridge, M., (2009). Can a *Beauveria bassiana* F418 transformant expressing green fluorescence be used to monitor the fate of this entomopathogenic fungus in soil? Poster presentation at the 3rd International Symposium on Biological Control of Arthropods, 8-13 February 2009, Christchurch, New Zealand.

2008 **Blond, C**., Ridgway, H., Glare, T., Condron, L. and Brownbridge, M., (2008) Effects of production media and fertilisers on persistence and pathogenicity of *Beauveria bassiana* F418 strain and a transformant *gfp* F418 tr1 in soil. Oral presentation at the New Zealand Microbiology Society 2008 Conference, 18-21 November 2008, Christchurch, New Zealand.

2008 **Blond, C**., Ridgway, H., Glare, T., Chapman, B., and Brownbridge, M., (2008) Effects of production media and fertilisers on persistence and virulence of *Beauveria bassiana* F418 strain and a transformant *gfp* F418 tr1 in soil. Oral presentation at the 41st Annual Meeting of the Society for Invertebrate Pathology, 3-7 August 2008, Warwick, UK.

Appendix A

Media, antibiotics and buffers preparation

SDA

Dissolve in 1 L of distilled water:

Sabouraud dextrose agar (Merck)	65g/L
(contains 1 % peptone, 4 % glucose and 1.5 % agar)	

Then autoclave at 121 °C at 15 psi fpr 15 min before pouring into Petri dishes.

10 % SDA

Dissolve in 1 L of distilled water:

Sabouraud dextrose agar (Merck)	6.5g/L
Agar	13.5g/L

Then autoclave at 121 °C at 15 psi for 15 min.

Tetracycline chloride 1.5 %

Dissolve 0.15 g of tetracycline chloride (Sigma-Aldrich, USA) in 10 mL of methanol (LabServ, Australia).

Can be stored at -20 °C.

Streptomycin sulphate 10 %

Dissolve 1.5 g of streptomycin sulphate (Sigma-Aldrich, USA) in 15 mL of filter sterilised distilled water.

Can be stored at -20 °C.

Cycloheximide 1.56 %

Dissolve 375 mg of cycloheximide (Sigma-Aldrich, USA) in 12 mL of methanol (LabServ, Australia). Once dissolved, add 12 mL of distilled water.

repared fresh before adding to the media using a sterile filter syringe.

10 % SDA+A

Before pouring into Petri plates, add to each L of 10 % SDA:

Tetracycline chloride 1.5 %	3.33 mL
Streptomycin sulphate 10 %	3.5 mL
Cycloheximide 1.56 % (filter sterilise)	8 mL

10 % SDA+A therefore contains 50 mg/l of tetracycline chloride, 350 mg/l of streptomycin sulphate and 125 mg/l of cycloheximide.

10 % SDA+A+Hyg

Before pouring into Petri dishes, add 4 mL of hygromycin B (50 mg/mL, Invitrogen, USA) to 10 % SDA+A described above.

PDA+A

Dissolve in 1 L of distilled water:

Potato dextrose agar (Merck)	39g/L
(contains % agar)	

Then autoclave at 121 °C at 15 psi for 15 min.

Before pouring into Petri plates, add to each L of PDA:

Tetracycline chloride 1.5 %	3.33 mL
Streptomycin sulphate 10 %	3.5 mL
Cycloheximide 1.56 % (filter sterilise)	8 mL

PDA+A therefore contains 50 mg/l of tetracycline chloride, 350 mg/l of streptomycin sulphate and 125 mg/l of cycloheximide.

0.01 % SDWA

Add 100 mg of agar to 1L of distilled water and autoclave at 121 °C at 15 psi for 15 min.

0.01 % Triton X-100

Dilute Triton X-100 (BDH, Poole, UK) to 0.01 % in distilled water and antoclave at 121 °C at 15 psi for 15 min.

0.5 M EDTA

To make 1 L of 0.5 M EDTA:

Disodium ethylediamnietetraacetate (Fisher scientific, UK)	186.12 g
H_2O	800 mL
Adjust pH to 8 with NaOH 9LabServ, Biolab, Australia).	
Adjust to 1 L with H_2O	

50 × TAE

To make 1 L of 50 × TAE:

Tris base (J.T. Baker, USA)	242 g
H_2O	500 mL
0.5 M EDTA (pH8)	100 mL
Acid acetic	57.1 mL
Adjust to 1 L with H_2O	

1 % agarose gel in TAE

Add 5 g of agar (Bioline, Australia) to 500 mL of 1 × TAE, mix and microwave until the agar is dissolved and homogene.

Appendix B

Sequencing of the *gfp-hph* cassette and outside the cassette

```
>
1           CATCGAGYMGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGAC
61          GGCCCCGWGCKGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGAC
121         CCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACT
181         CACGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCCCGGCTGCAGATCGTTCAAACATT
241         TGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAA
301         TTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATG
361         AGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAA
421         ATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCCG
481         ATGATAAGCTGTCAAACATGAGAATTCGTAATCATGTCATAGCTGTTTCCTGTGTGAAAT
541         TGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGG
601         GGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCA
661         GTCGGGGAAACCTGTCGTGCCAGCTGCWTTAATGAATCGGCCAACGCGCGGGGAGGAGGC
721         GGTTTGCGTATTGGCTAGAGGCAGCTTGCCATCGAGTTTCTCCATAATAATGTGTGAGTA
781         GTTCCCAGATAAGGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAG
841         AAACCCTTRGTRTRTATTTGTATTTKKAAAATACTTCYATCAATAAAATTTCTAATTCCT
901         AAAACCAAAATCCAGTACTAAAATCCAGATCCCCCGAATTAATTCGGCGTTAATTCAGTA
961         CATTAAAAACGGCCGCAATGWGTTATTAAGTTGTCTAAGCGTCAATTTGTTTACACCACA
1021        ATA 1023
```

sGFP partial gene

NOS terminator

CaMV35S

Figure B.1 Common sequence (1023 bp) for F418 *gfp* tr1 and F418 *gfp* tr3 in the *gfp-hph* cassette with partial sGFP gene, NOS terminator and CaMV35S polyA underlined in green, yellow and blue respectively.

```
1           GACGTAGCCAGCTTCGGCCTCTGCTTTTTCTACGACAATATTAGCCACATGGTCCGAGTC
61          GGATATATTTGCGCGTGAATCGCACCTTTCGCGGGAACTTTATAGTGAACGACATAGTCG
121         ACACTGTTTTTGGGGAAGAACAGTTAGCGCAGCTGTCAGTAAGCGGGGAACAGATGGCTG
181         AGAGGAGACGATACCCATTGTTGTCGTCGACACTGCCATCATTCTTTGGAGAAGCCATTG
241         CGGTCGCGGTGGTTTCCATCATGGGCCAAACATCGATGTGAAGAAGTGAAGATGAGGGGA
301         ACGAGACAGTGATTTGGGGTCGAAGCGAGATGACGTGGTGGAGGGAGCTACGGACAGGGG
361         GGGCAGCTGGAGATGGAAGCTGTTGTGGAGTTTTGTTTCAGCTGCAAGAATCTCTCTCTA
421         CCTTAGTGTATCAGACGTCGACTGCCGCCTTGCAAGAGAAGATGACAAGAGCACATAAAA
481         TTGCAAAATATGAAAGTAGAAACAGCGTGAATACTACATCTTAAAATTTTTCTTCGCCAT
541         GCCAATGAGAAGAATATTAGTTCAAAACCTAGGCAAAGAGGCATGGTTAGGCACCATGAC
601         GCCGTGACTAAATACCTAGACACGGCCAGGTAGCTGAGAGGTGCTTGTGTGGGGCGCCAA
661         CAGAGCTGAGATGGCTGCAACCCTGGCAGGCACTGAGCGCATCAGATACTTGCACCGCCT
721         CTCTGTTGAGACTGTTGACTGCTCATGACTAGGCAGCTTTCGAAGAAGCAGACAATATTT
781         TATAAAGGGCCGGGGACGCAGGATCGAGGTAAGCTGAGCGCAAGCCTCACGTTCCAGAGA
841         CGCCACAGTGTACAAAGTGACGGTGCTCCCTCCTCTAACCAAGACGATCGCCACCGGGGC
901         AGCCACACCAGCTATAGTGCGCACACTTGATCTACTGATTCGCGATTAGAGCTCCACAGT
961         GCTACTCGGAAACCGCCGCTTAAGATGTGGAGCGAGGTGACAGAGATCGTCAAGCAAGTT
1021        CGAAGACTGGATGATATCGAATGAATCGCC 1050
```

Figure B.2 Sequence (1050 bp) from F418 *gfp* tr1 outside the *gfp-hph* cassette

```
1           TATCCTGGAGTACGCTCGACAAACGGACAGCATCTTGTTCCTGCTGATACACCCCCCCCT
61          CTCACAAAAAAACCCCCATGAACGCCCCCTCCCCCCCAGAACCTGGCCAAGTTGACCGGC
121         CTCTTCACTTGCTCTCGTCTCTCACTACACGCCCAAAAAAACAAAACCATCCCCGGCCCA
181         AGCTCCAGCTTCTTGTAGGACCTCCTCTCCTCTCTCTCTACTTTTCTTCTAACCACCCGT
241         CGTTTTAAACCATTGTCCGACTCGGTCTTCAGAAGCATCTTTTCCTCACTCCCTCTTTTC
301         TTTTATAACCGCTTCTTCTTCCCATCCTAAAACCTTGAACCCTCCTCCGCCGCCGAGACC
361         CCCCCTCTCTCCCCTTGGCCCATCACCACCCGCTTACCGACCTCTCCATTTTTTCGCCAA
421         TTCTCAGACTCTGTTTCGAGACGGCAGGAAGGAAGAAAAGAAAGAAAACGGTATGGCTTC
481         CTCTTTTTACCCCCTTTTTCATTTTTTAATGTTGCTTGCTTTTACTGCCAAATCCACAAC
541         GGTTCCTGTCTCACTCAATTCCTCGCCATCGTGCATGTTGGTTGATGCGCTGCGCCGCCA
601         TCCTCTGCCCTCTTGACTCTTGCCCGCTTTGCACCACCACCACCACCACGTCGTGTACTT
661         ACTTGCTTCTGCCCTGGTCAGCGCACCCAGCCTTCATCTATGTCGGACTAATAG 714
```

Figure B.3 Sequence (714 bp) from F418 *gfp* tr3 outside the *gfp-hph* cassette

Appendix C
Statistical tables for Chapter 2

Table C.1 Analysis of variance (ANOVA) of F418, F418 *gfp* tr1 and F418 *gfp* tr3 germination rate (Log_{10}) after incubation at different temperatures and times in two different experiments (May and July 2009).

Source of variation		d.f.	s.s.	m.s.	F	P
experiment.temperature stratum	experiment	1	0.124716	0.124716	0.98	0.426
	temperature	2	4.329978	2.164989	17.07	0.055
	Linear cpt	1	0.077807	0.077807	0.61	0.515
	Residual	2	0.253625	0.126813	0.84	
experiment.temperature .incubation stratum	incubation	1	10.288443	10.288443	67.96	0.004
	Linear cpt	1	10.288443	10.288443	67.96	0.004
	Temperature.incubation	2	4.202543	2.101272	13.88	0.030
	Lin.lin cpt	1	0.081228	0.081228	0.54	0.517
	Residual	3	0.454193	0.151398	25.42	
experiment.temperature .incubation.units stratum	replicates	2	0.030458	0.015229	2.56	0.085
	fungus	2	0.449657	0.224828	37.75	<.001
	Temperature.fungus	4	0.176732	0.044183	7.42	<.001
	Linear cpt	2	0.007657	0.003829	0.64	0.529
	incubation.fungus	2	0.457135	0.228568	38.38	<.001
	Linear cpt	2	0.457135	0.228568	38.38	<.001
	Temperature.incubation. fungus	4	0.177485	0.044371	7.45	<.001
	Linear cpt	2	0.011090	0.005545	0.93	0.399
	residual	69	0.410910	0.005955		
	total	94	17.075780			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.2 ANOVA of F418, F418 *gfp* tr1 and F418 *gfp* tr3 colony growth (mm/d) after 15 d incubation at 15, 20 and 25 °C in two different experiments (May and June 2009).

Source of variation	d.f.	s.s.	m.s.	F	P
rep	5	0.04667	0.00933	0.43	0.826
experiment	1	1.84238	1.84238	84.96	<.001
fungus	2	0.79460	0.39730	18.32	<.001
temperature	2	41.61822	20.80911	959.61	<.001
Linear cpt	1	40.82937	40.82937	1882.84	<.001
Experiment.fungus	2	0.06154	0.03077	1.42	0.249
Experiment.temperature	2	2.22881	1.11441	51.39	<.001
Linear cpt	1	1.74216	1.74216	80.34	<.001
Fungus.temperature	4	0.79366	0.19841	9.15	<.001
Linear cpt	2	0.08424	0.04212	1.94	0.151
Experiment.fungus.temperature	4	0.99491	0.24873	11.47	<.001
Linear cpt	2	0.26372	0.13186	6.08	
residual	70	1.51795	0.02168		
total	92	41.86435			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.3 ANOVA of F418, F418 *gfp* tr1 and F418 *gfp* tr3 conidia production $\log_{10}$ (10 mm Ø SDA disc) after 15 d incubation at 15 and 20 °C in two different experiments (May and June 2009).

Source of variation	d.f.	s.s.	m.s.	F	P
rep	2	0.15873	0.07937	2.01	0.157
experiment	1	2.81355	2.81355	71.41	<.001
fungus	2	0.00539	0.00270	0.07	0.934
temperature	1	2.38049	2.38049	60.42	<.001
Linear cpt	1	2.38049	2.38049	60.42	<.001
Experiment.fungus	2	0.00554	0.00277	0.07	0.932
Experiment.temperature	1	0.86004	0.86004	21.83	<.001
Linear cpt	1	0.86004	0.86004	21.83	<.001
Fungus.temperature	2	0.10206	0.05103	1.30	0.294
Linear cpt	2	0.10206	0.05103	1.30	0.294
Experiment.fungus.temperature	2	0.09543	0.04772	1.21	0.317
Linear cpt	2	0.09543	0.04772	1.21	0.317
residual	22	0.86678	0.03940		
total	35	7.28803			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.4 ANOVA of F418, F418 *gfp* tr1 and F418 *gfp* tr3 conidia production $\log_{10}$ (10 mm Ø SDA disc) after 15 d incubation at 25 °C in two different experiments (May and June 2009).

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	2	0.12439	0.06220	3.66	0.125
fungi	2	0.17453	0.08727	5.14	0.078
residual	4	0.06793	0.01698		
total	8	0.36686			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.5 ANOVA of F418, F418 *gfp* tr1 and F418 *gfp* tr3 conidia size after 15 d incubation at 15 and 20 °C in two different experiments (May and June 2009).

Source of variation	d.f.	s.s.	m.s.	F	P
rep	2	0.02825	0.01412	0.83	0.450
experiment	1	1.16461	1.16461	68.28	<.001
fungus	2	0.00334	0.00167	0.10	0.907
temperature	1	0.01715	0.01715	1.01	0.327
Linear cpt	1	0.01715	0.01715	1.01	0.327
Experiment.fungus	2	0.01565	0.00783	0.46	0.638
Experiment.temperature	1	0.04956	0.04956	2.91	0.102
Linear cpt	1	0.04956	0.04956	2.91	0.102
Fungus.temperature	2	0.06982	0.03491	2.05	0.153
Linear cpt	2	0.06982	0.03491	2.05	0.153
Experiment.fungus.temperature	2	0.11569	0.05785	3.39	0.052
Linear cpt	2	0.11569	0.05785	3.39	0.052
residual	22	0.37523	0.01706		
total	35	1.83932			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.6 ANOVA of F418, F418 *gfp* tr1 and F418 *gfp* tr3 conidia size after 15 d incubation at 25 °C in two different experiments (May and June 2009).

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	2	0.034025	0.017013	1.94	0.258
fungi	2	0.040707	0.020354	2.32	0.215
residual	4	0.035158	0.008790		
total	8	0.109891			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.7 ANOVA of F418, F418 *gfp* tr1 and F418 *gfp* tr3 virulence (Log_{10} LT_{50} in days) against *T. molitor* larvae using 10^4 and 10^6 conidia/mL (May 2009).

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	5	0.0047429	0.0009486	2.47	0.059
fungi	2	0.0006706	0.0003353	0.87	0.429
Concentration	1	0.2585996	0.2585996	674.39	<.001
Linear cpt	1	0.2585996	0.2585996	674.39	<.001
concentration × fungi	2	0.0001360	0.0000680	0.18	0.838
Linear cpt	2	0.0001360	0.0000680	0.18	0.838
residual	25	0.0095864	0.0003835		
total	35	0.2737355			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.8 ANOVA of virulence of single and mixed suspensions (different ratio) of F418 and F418 *gfp* tr1 (Log_{10} LT_{50} in days) against *T. molitor* larvae using 10^5 conidia/mL.

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	2	0.006014	0.003007	0.37	0.704
treatment	4	0.029468	0.007367	0.90	0.507
residual	8	0.065516	0.008189		
total	14	0.100998			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.9 ANOVA of virulence of single and mixed suspensions (different ratio) of F418 and F418 *gfp* tr1 (Log_{10} LT_{50} in days) against *T. molitor* larvae using 10^7 conidia/mL.

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	2	0.0008053	0.0004026	1.01	0.406
treatment	4	0.0006626	0.0001657	0.42	0.793
residual	8	0.0031839	0.0003980		
total	14	0.0046518			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.10 ANOVA of conidia production (Log_{10}) on *T. molitor* inoculated with single or mixed suspensions (different ratio) of F418 and F418 *gfp* tr1 at 10^5 conidia/mL.

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	4	2.7952	0.6988	1.44	0.241
block	2	1.2208	0.6104	1.26	0.296
treatment	4	0.1942	0.0486	0.10	0.982
block x treatment	8	4.6323	0.5790	1.20	0.330
residual	34	16.4593	0.4841		
total	52	22.0844			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.11 ANOVA of conidia production (Log_{10}) of *T. molitor* inoculated with single or mixed suspensions (different ratio) of F418 and F418 *gfp* tr1 at 10^7 conidia/mL.

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	5	1.3381	0.2676	0.55	0.736
block	2	1.4831	0.7415	1.53	0.224
treatment	4	0.1195	0.0299	0.06	0.993
block x treatment	8	3.7840	0.4730	0.98	0.463
residual	69	33.4657	0.4850		
total	88	40.0951			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.12 ANOVA of virulence of single and mixed suspensions (different ratio) of F418 and F418 *gfp* tr3 (Log_{10} LT_{50} in days) against *T. molitor* larvae using 10^5 conidia/mL.

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	2	0.005568	0.002784	0.48	0.637
treatment	4	0.013457	0.003364	0.58	0.687
residual	8	0.046585	0.005823		
total	14	0.065610			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.13 ANOVA of virulence of single and mixed suspensions (different ratio) of F418 and F418 *gfp* tr3 (Log_{10} LT_{50} in days) against *T. molitor* larvae using 10^7 conidia/mL.

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	2	0.002444	0.001222	0.93	0.432
treatment	4	0.004551	0.001138	0.87	0.523
residual	8	0.010482	0.001310		
total	14	0.017478			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.14 ANOVA of conidia production (Log_{10}) of *T. molitor* inoculated with single or mixed suspensions (different ratio) of F418 and F418 *gfp* tr3 at 10^5 conidia/mL.

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	5	4.0178	0.8036	3.15	0.014
block	2	1.3182	0.6591	2.59	0.085
treatment	4	1.3022	0.3256	1.28	0.290
block x treatment	8	2.8076	0.3510	1.38	0.228
residual	53	13.4991	0.2547		
total	72	20.9824			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.15 ANOVA of conidia production (Log_{10}) of *T. molitor* inoculated with single or mixed suspensions (different ratio) of F418 and F418 *gfp* tr3 at 10^7 conidia/mL.

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	4	8.4946	2.1237	3.85	
block	2	0.7568	0.3784	0.69	0.511
treatment	4	9.7789	2.4447	4.43	0.006
residual	33	18.1979	0.5515		
total	43	27.8241			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.16 ANOVA of F418, F418 *gfp* tr1 and F418 *gfp* tr3 virulence (Log_{10} of LT_{50} in days) against small and large larvae of *T. molitor* using 10^4 and 10^5 conidia/mL.

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	2	0.001025	0.000513	0.22	0.803
fungi	2	0.005585	0.002792	1.21	0.321
concentration	1	0.225975	0.225975	97.60	<0.001
insect size	1	0.000548	0.000548	0.24	0.632
fungus × concentration	2	0.010468	0.005234	2.26	0.132
fungus × insect size	2	0.000513	0.000257	0.11	0.896
concentration × insect size	1	0.019857	0.019857	8.58	0.009
fungus × concentration× insect size	2	0.009927	0.004963	2.14	0.145
residual	19	0.043992	0.002315		
total	32	0.285922			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.17 ANOVA of F418, F418 *gfp* tr1 and F418 *gfp* tr3 virulence (Log_{10} of LC_{50}) against small and large larvae of *T. molitor.*

Source of variation	d.f.	s.s.	m.s.	F	P
replicates	2	0.0574	0.0287	0.18	0.841
fungi	2	0.1351	0.0676	0.42	0.671
insect size	1	0.3362	0.3362	2.07	0.181
fungi x insect size	2	0.0912	0.0456	0.28	0.761
residual	10	1.6271	0.1627		
total	17	2.2470			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table C.18 ANOVA of F418, F418 *gfp* tr1 and F418 *gfp* tr3 conidia production on small and large larvae of *T. molitor* using 10^4 and 10^5 conidia/mL.

Source of variation		d.f.	s.s.	m.s.	v.r.	F pr.
Replicate stratum		3	4.1682	1.3894	3.74	
Replicate.units stratum	Block	2	2.1560	1.0780	2.90	0.063
	Fungus	2	2.9613	1.4806	3.98	0.024
	concentration	1	0.0104	0.0104	0.03	0.868
	Insect size	1	7.0788	7.0788	19.04	<.001
	Fungus.concentration	2	3.0038	1.5019	4.04	0.023
	Fungus.insect size	2	0.0414	0.0207	0.06	0.946
	Concentration.insect size	1	0.0420	0.0420	0.11	0.738
	Fungus.dose.insect size	2	1.9262	0.9631	2.59	0.084
	Residual	56	20.8217	0.3718		
	Total	72	31.2201			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Appendix D
Templeton soil loam soil analyses

The soil analyses were done by NZlabs (Hamilton, NZ).

Templeton silt loam texture:

Coarse sand % 0.6-2 mm	Medium sand % 0.2-0.6 mm	Fine sand % 0.06-2 mm	Silt %	Clay %
0.2 %	2.3 %	16.5 %	57.5 %	23.5 %

Templeton silt loam soil analysis:

	Pasteurised soil	Fresh soil collected April 2008 analysed July 2008	Fresh soil collected May 2010 Analysed May 2011	Fresh soil collected July 2009 analysed May 2011
pH	5.4	5.7	6.1	6.3
Ca calcium	8	8	10	14
P Olsen phosphate	32	12	8	32
K potassium	2	3	2	5
$S(SO_4)$ sulphate sulphur ppm	45	21	18	25
Mg magnesium	11	14	14	20
Na sodium	8	8	11	14
OC organic carbon %	3	3	3	4.1
OC organic matter %	5.2	5.2	5.2	7
NH_4-N ammonium nitrogen	36	2	2	7
NO_3-N nitrate nitrogen	10	44	39	253
Moisture content %	<1	17	10	5
Pseudo total calcium %	0.47	0.47		
Mineral N ppm			41	260

Appendix E

Finding a non-host insect to *B. bassiana* F418

Testing F418 against Porina, *Wiseana* spp. (Lepidoptera: Hepialidae)

Pathogenicity bioassay was carried out against field collected Porina, *Wiseana copularis and W. cervinata* late instar (7, 8 or 9th instar) larvae (Colin Ferguson, AgResearch, Invermay, NZ). Batches of 5 *Wiseana* spp. larvae were immersed for 10 s in 150 mL F418 conidial suspension at 2 × 10^5 conidia/mL, 2 × 10^7 conidia/mL or in 150 mL of 0.01 % Triton X-100 (BDH, Poole, UK) as a control. The larvae were separated in 5 blocks with 3 larvae inoculated with 2 × 10^5 conidia/mL suspension, 3 larvae inoculated with 2 × 10^7 conidia/mL suspension and 4 control larvae in each block. Each larva was placed in a well filled with moist peat and fed a piece of carrot (2 mm × 2 mm) every few days. Larvae were arranged in a RCBD at 15 °C incubator. Larvae were checked daily for 5 weeks for mortality.

The mortality rate due to F418 on *Wiseana* spp. larvae was 13.3 % with 2 × 10^5 conidia/mL and 33.3 % with 2 × 10^7 conidia/mL.

Testing F418 against *Spodoptera litura* F. (Lepidoptera: Noctuidae)

Pathogenicity bioassay was carried out against laboratory reared *Spodoptera litura* 5th instar larvae (Insect Rearing Facility, HortResearch, now Plant and Food, Auckland, NZ). F418 suspension of 1 × 10^5 conidia/mL and 1 × 10^7 conidia/mL were prepared in 0.01 % Triton X-100 and 48 larvae were inoculated with 10 µL of 1 × 10^5 conidia/mL, 48 larvae were inoculated with 1 × 10^7 conidia/mL and 72 larvae were treated with 10 µL of 0.01 % Triton X-100 as a control. Larvae were held individually in a well of a 24 well plates, fed with a piece (5 mm × 5 mm) of lima bean diet (McManus & Burgess, 1995) every few days and incubated at 15 °C. Larvae were checked every 3 d over 24 d for mortality.

There was no *S. litura* mortality observed due to F418 with any of the treatments with F418 or control.

Testing F418 against *Costelytra zealandica* (Coleoptera: Scarabaeidae)

Pathogenicity bioassay was carried out against field collected *Costelytra zealandica* 3rd instar larvae (Richard Townsend, AgReseach, Lincoln, NZ). F418 suspension of 1.27×10^8 conidia/mL was prepared in 0.01 % Triton X-100 and 48 larvae were treated with 10 µL of the F418 suspension and 48 larvae were inoculated with 10 µL of 0.01 % Triton X-100 as control. Half of the treated and control larvae were held individually in wells containing fresh soil while the other half was held in wells without soil. A piece of carrot (2 mm × 2 mm) was given to each larva at the start of the experiment. Larvae were incubated at 15 °C and checked every 3 d for 5 weeks for mortality.

There was no *C. zealandica* mortality observed due to F418 in the treatment or control.

Summary

Wiseana spp. could not be used as a non-host as F418 is pathogenic to this insect. Both *S. litura* and *C. zealandica* larvae could be used as non-host insect for F418 as F418 is not pathogenic to either insect. Although, *S. litura* only dig a burrow in the soil to hide when not eating and feed above the soil surface whereas *C. zealandica* larvae do spend the whole time in the soil, moving through it and feeding of roots inisde the soil. Therefore, due to the life cycle, *C. zealandica* is a better non-host for studies with non-host insect and F418 in the soil.

Appendix F

Virulence of *B. bassiana* F418 in pasteurised soil under different soil conditions and correlation to persistence

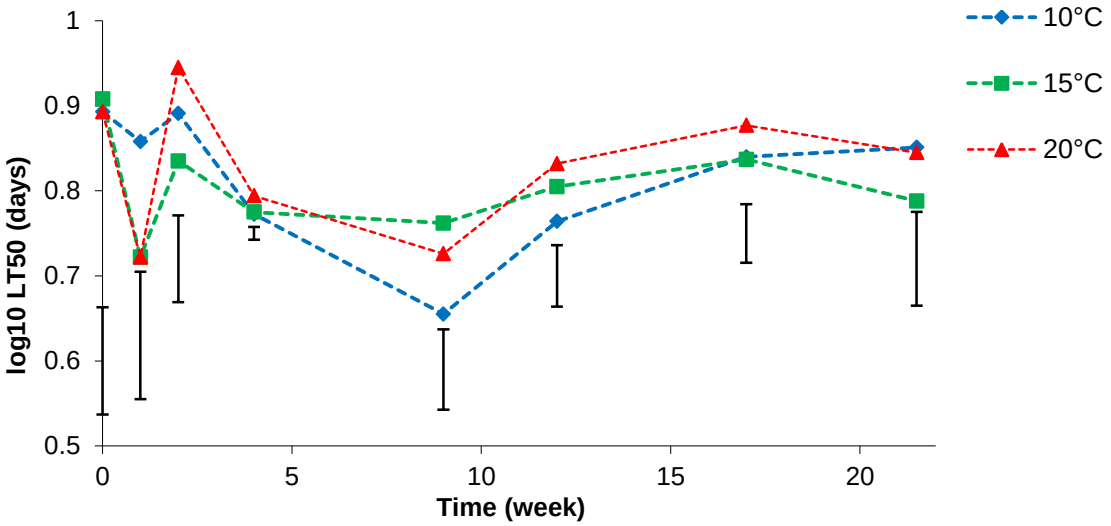

Figure F.1 Virulence (log_{10} of LT_{50} in days) against *Tenebrio molitor* larvae of F418 in pasteurised soil at 10, 15 and 20 °C over 21.5 weeks at 22 % GWC. Vertical bars are LSD (P < 0.05).

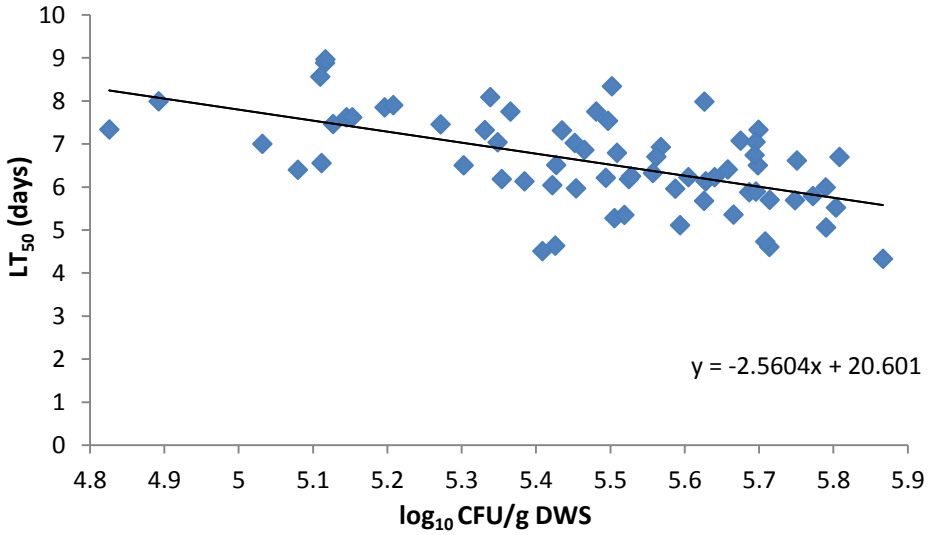

Figure F.2 Linear correlation between virulence (LT_{50} in days) against *Tenebrio molitor* larvae and log_{10} of CFU/g DWS of F418 in pasteurised soil at 10, 15 and 20 °C over 21.5 weeks (P < 0.001).

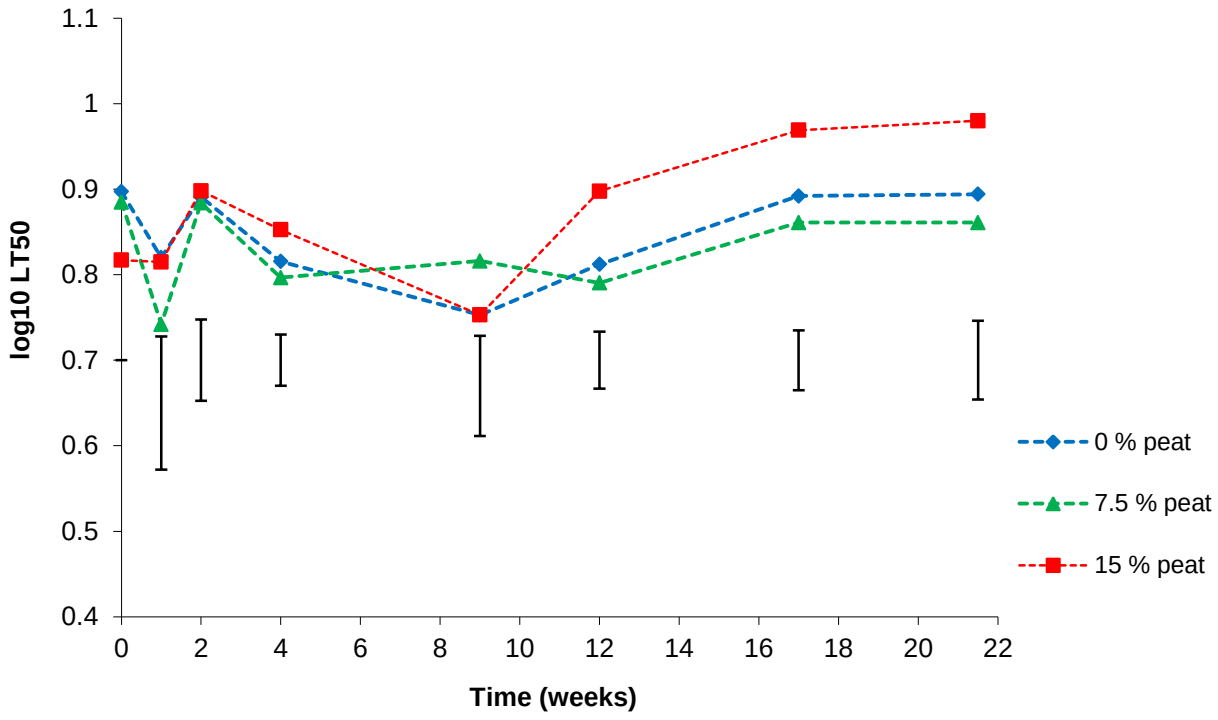

Figure F.3 Virulence (log_{10} of LT_{50} in days) against *Tenebrio molitor* larvae of F418 in pasteurised soil with 0, 7.5 and 15 % peat over 21.5 weeks at 22 % GWC and 15 °C. Vertical bars are LSD ($P < 0.05$).

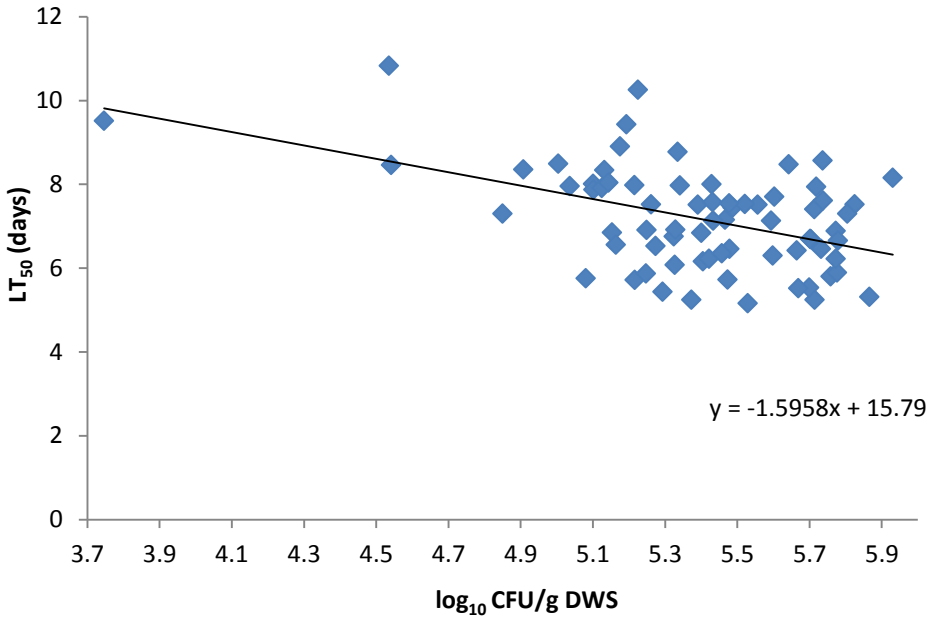

Figure F.4 Linear correlation between virulence (LT_{50} in days) against *Tenebrio molitor* larvae and log_{10} of CFU/g DWS of F418 in pasteurised soil with 0, 7.5 and 15 % peat over 21.5 weeks at 22 % GWC and 15 °C ($P = 0.003$).

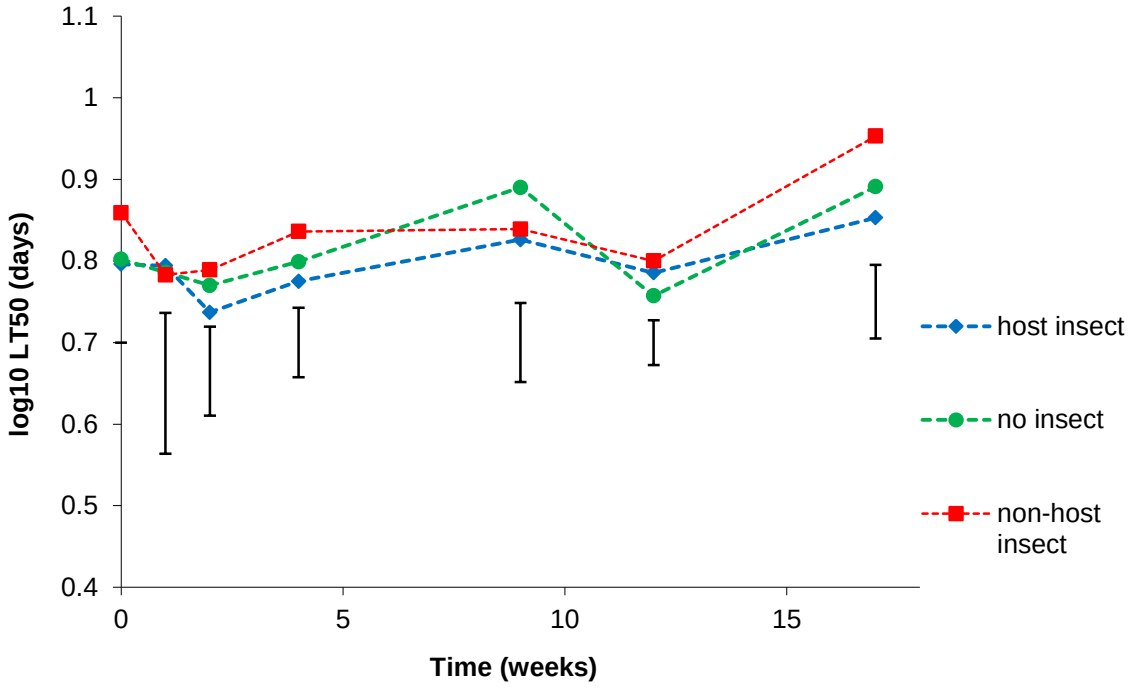

Figure F.5 Virulence (log_{10} of LT_{50} in days) against *Tenebrio molitor* larvae of F418 in pasteurised soil with host, non-host and no insect over 17 weeks at 22 % GWC and 15 °C. Vertical bars are LSD (P < 0.05).

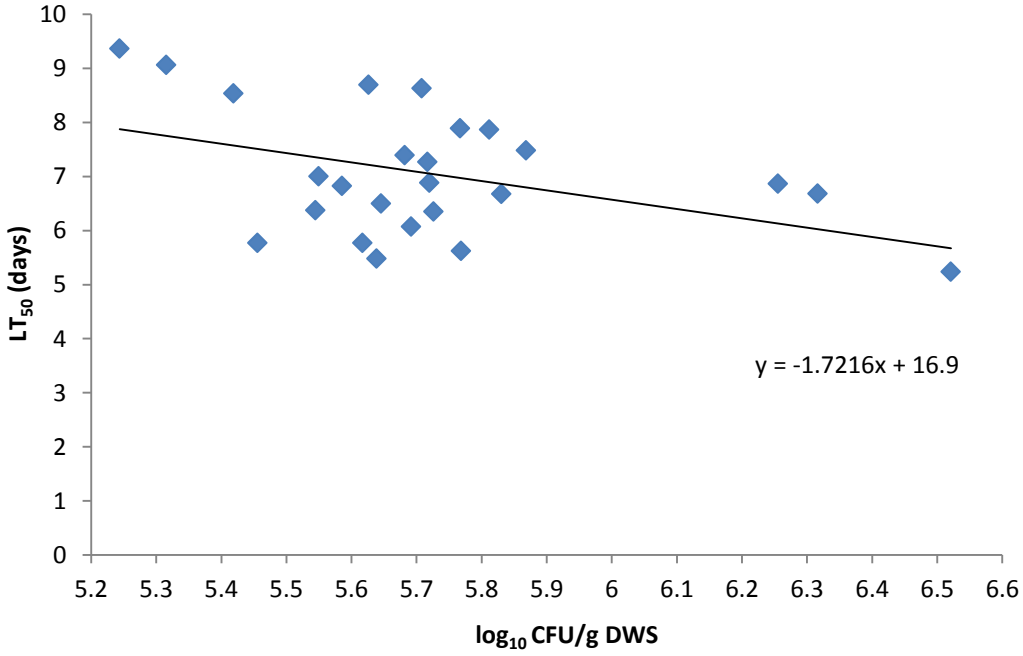

Figure F.6 Linear correlation between virulence (LT_{50} in days) against *Tenebrio molitor* larvae and log_{10} of CFU/g DWS of F418 in pasteurised soil with host, non-host and no insect between 9 and 17 weeks at 22 % GWC and 15 °C (P = 0.033).

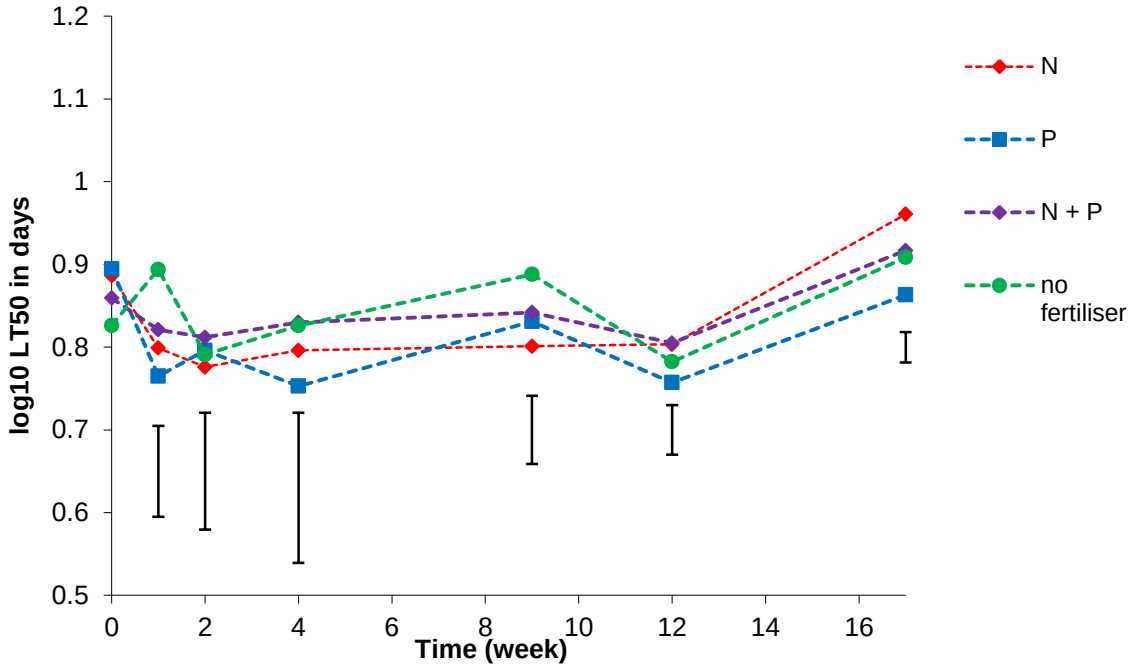

Figure F.7 Virulence (log_{10} of LT_{50} in days) against *Tenebrio molitor* larvae of F418 in pasteurised soil amended with superphosphate (P), urea (N), both fertiliser (N+P) or no fertiliser over 17 weeks at 22 % GWC and 15 °C. Vertical bars are LSD ($P < 0.05$).

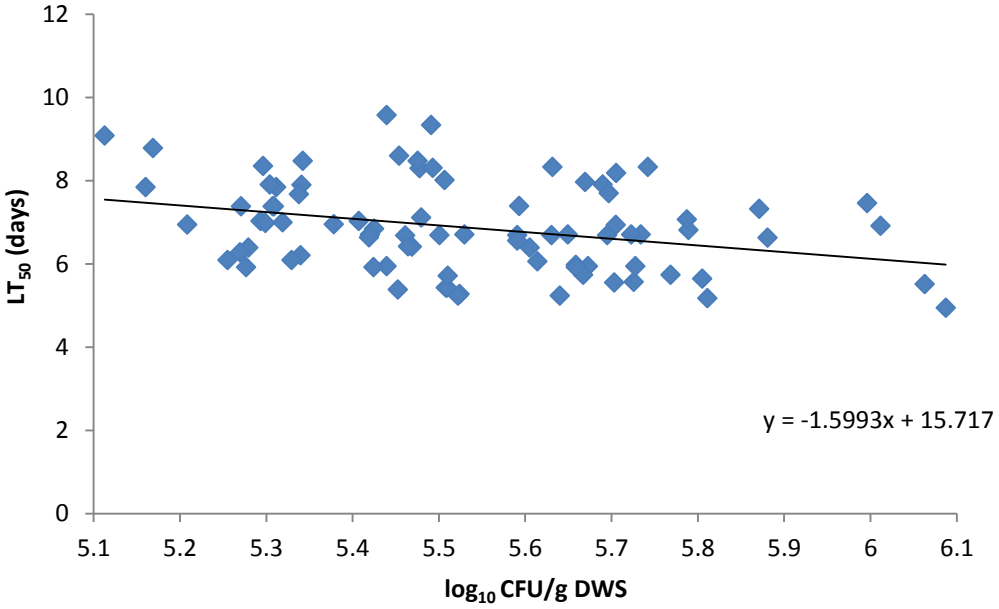

Figure F.8 Linear correlation between virulence (LT_{50} in days) against *Tenebrio molitor* larvae and log_{10} of CFU/g DWS of F418 in pasteurised soil amended with superphosphate (P), urea (N), both fertiliser (N+P) or no fertiliser over 17 weeks at 22 % GWC and 15 °C ($P = 0.003$).

Appendix G

Effect of some pre-application and soil factors on *B. bassiana* F418 persistence in pasteurised soil

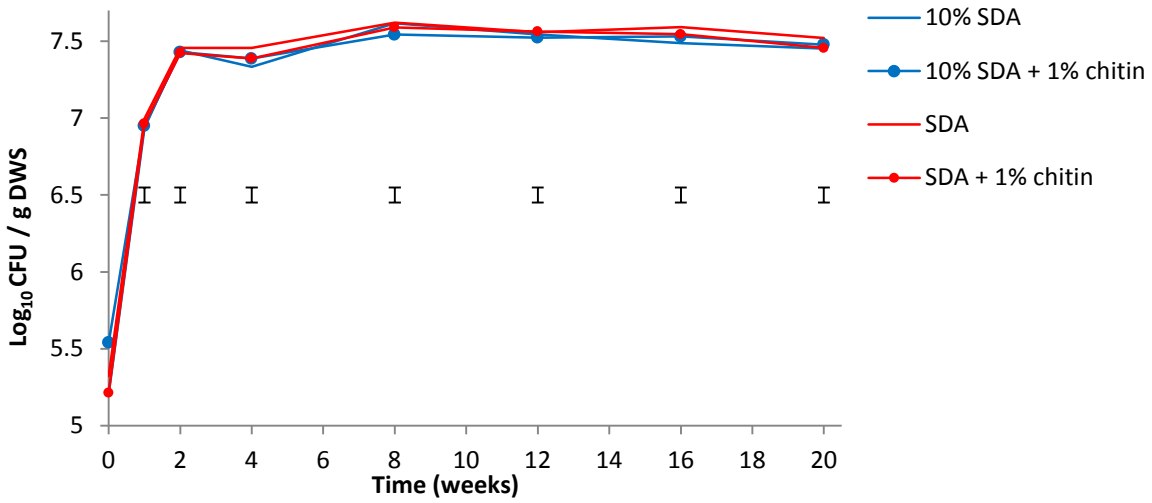

Figure G.1 F418 population recovered (log_{10} CFU/g DWS) from pasteurised soil at 28 % GWC over 20 weeks of incubation at 15 °C. F418 was grown on different media: 10 % SDA, 10 % SDA+1 % chitin, SDA or SDA+1 % chitin (Appendix A). Vertical bars are LSD ($P < 0.05$). Pasteurised soil was warm (>30 °C) when mixed with the fungal suspension. The experiment was set up in February 2008.

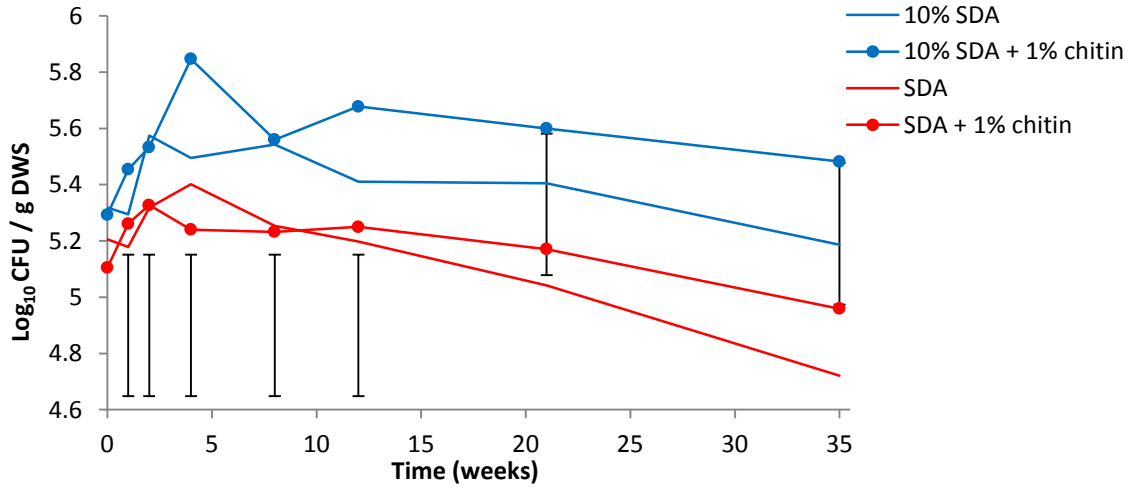

Figure G.2 F418 population recovered (log_{10} CFU/g DWS) from pasteurised soil at 28 % GWC over 35 weeks of incubation at 15 °C. F418 was grown on different media: 10 % SDA, 10 % SDA+1 % chitin, SDA or SDA+1 % chitin (Appendix A). Vertical bars are LSD ($P < 0.05$). Pasteurised soil was at 22 ± 2 °C when mixed with the fungal suspension. The experiment was set up in April 2008.

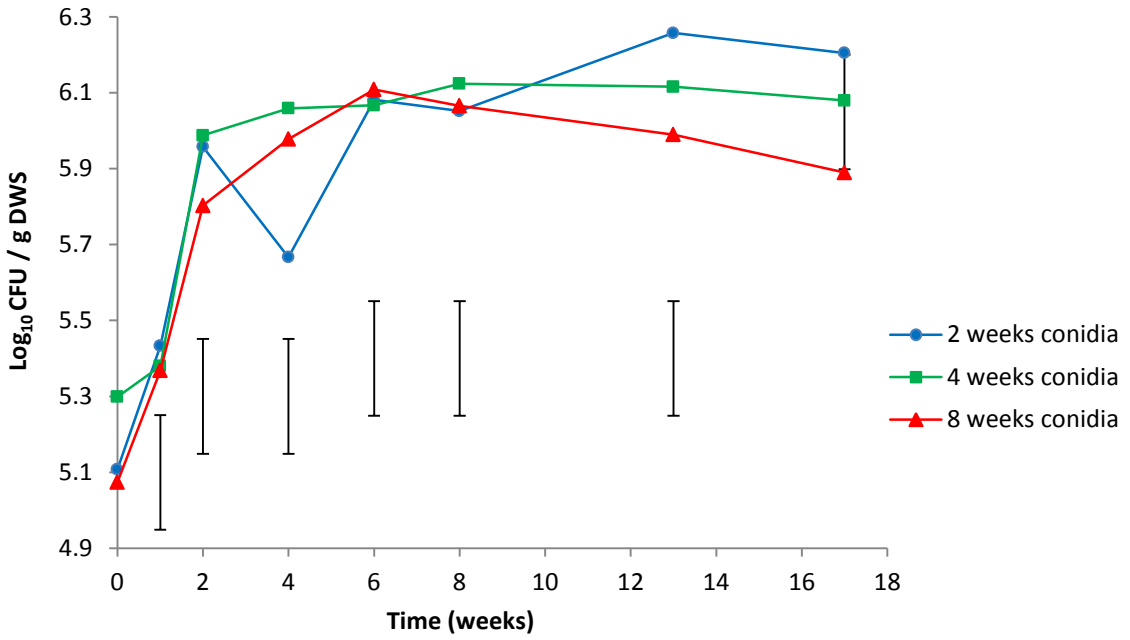

Figure G.3 F418 population recovered (log_{10} CFU/g DWS) from pasteurised soil at 28 % GWC over 17 weeks of incubation at 15 °C. F418 conidia were from culture incubated for different length of time: 2, 4 or 8 weeks. Pasteurised soil was at 22 ± 2 °C when mixed with the fungal suspension. Vertical bars are LSD (P < 0.05). The experiment was set up in February 2008.

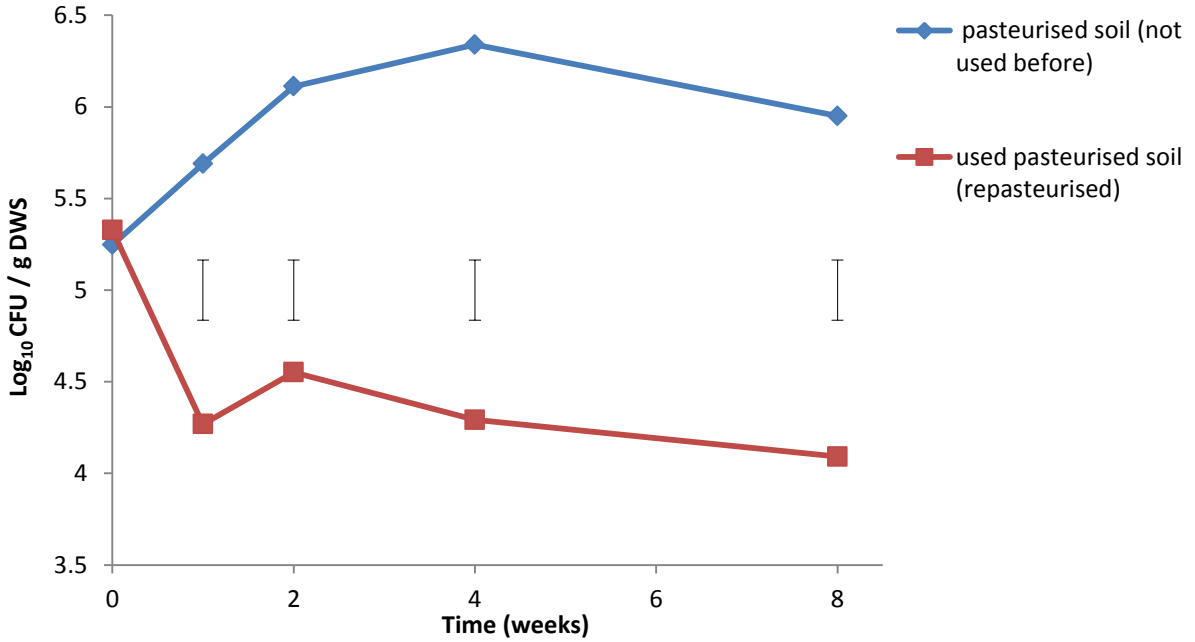

Figure G.4 F418 population recovered (log_{10} CFU/g DWS) from pasteurised soil at 28 % GWC over 8 weeks of incubation at 15 °C. The pasteurised soil was either not used before soil or used soil in previous experiment, repasteurised. Pasteurised soil was at 22 ± 2 °C when mixed with the fungal suspension. Vertical bars are LSD (P < 0.05). The experiment was set up in April 2008.

Appendix H

Statistical tables for Chapter 3

Table H.1 ANOVA of F418 CFU/g DWS (log_{10}) in pasteurised soil at 14, 22 and 31 % GWC over 21.5 weeks at 15 °C in laboratory microcosms.

Source of variation		d.f.	s.s.	m.s.	F	P
Replicate.moisture stratum	replicate	2	0.14727	0.07363	0.91	0.471
	moisture	2	8.24612	4.12306	51.17	0.001
	Linear cpt	1	7.94577	7.94577	98.61	<0.001
	residual	4	0.32232	0.08058	2.38	
Replicate.moisture.time stratum	time	7	2.28843	0.32692	9.64	<0.001
	Linear cpt	1	0.23633	0.23633	6.97	0.012
	Moisture.time	14	3.40822	0.24344	7.18	<0.001
	Linear cpt	1	2.49377	2.49377	73.52	<0.001
	residual	42	1.42455	0.03392		
	Total	71	15.83691			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table H.2 ANOVA of F418 virulence (Log_{10} LT_{50} in days) data from pasteurised soil at 14, 22 and 31 % GWC suspensions against *T. molitor* larvae.

Source of variation	d.f.	s.s.	m.s.	F	P
replicate	2	0.000700	0.000350	0.21	0.808
treatment	2	0.147516	0.073758	45.17	<.001
time	7	0.658958	0.094137	57.65	<.001
Treatment × time	14	0.044885	0.003206	1.96	0.044
residual	46	0.075110	0.001633		
total	71	0.927169			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table H.3 ANOVA of F418 CFU/g DWS (log_{10}) in pasteurised soil at 22 % GWC at 10, 15 and 20 °C over 21.5 weeks in laboratory microcosms.

Source of variation		d.f.	s.s.	m.s.	F	P
Replicate.temperature stratum	replicate	2	0.02135	0.01067	0.11	0.899
	temperature	2	0.19375	0.09687	1.00	0.445
	Linear cpt	1	0.01023	0.01023	0.11	0.762
	residual	4	0.38871	0.09718	7.64	
Replicate.temperature.time stratum	Time	7	2.14186	0.30598	24.06	<0.001
	Linear cpt	1	0.31219	0.31219	24.55	<0.001
	Temperature.time	14	0.72347	0.05168	4.06	<0.001
	Linear cpt	1	0.20453	0.20453	16.09	<0.001
	residual	38	0.48316	0.01271		
	Total	67	3.84674			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table H.4 ANOVA of F418 CFU/g DWS (log_{10}) in pasteurised soil at 22 % GWC with 0, 7.5 and 15 % peat content over 21.5 weeks at 15 °C in laboratory microcosms.

Source of variation		d.f.	s.s.	m.s.	F	P
Replicate.peat stratum	replicate	2	0.00804	0.00402	0.20	0.825
	peat	2	2.68357	1.34179	67.23	<0.001
	Linear cpt	1	0.32158	0.32158	16.11	0.016
	residual	4	0.07983	0.01996	1.12	
Replicate.peat.time stratum	Time	7	2.85884	0.40841	22.84	<0.001
	Linear cpt	1	0.71796	0.71796	40.16	<0.001
	peat.time	14	2.53458	0.18104	10.13	<0.001
	Linear cpt	1	1.41372	1.41372	79.08	<0.001
	residual	42	0.75085	0.01788		
	Total	67	8.91572			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table H.5 ANOVA of F418 CFU/g DWS (log_{10}) in pasteurised soil at 22 % GWC with host, non-host or no insect over 17 weeks at 15 °C in laboratory microcosms.

Source of variation		d.f.	s.s.	m.s.	F	P
Replicate.insect stratum	replicate	2	0.18371	0.09185	0.30	0.759
	insect	2	0.80387	0.40194	1.30	0.368
	residual	4	1.24095	0.31024	8.11	<0.001
Replicate.insect.time stratum	Time	6	2.38561	0.39760	10.39	<0.001
	Linear cpt	1	1.11778	1.11778	29.22	<0.001
	insect.time	12	7.03909	0.58659	15.34	<0.001
	Linear cpt	2	2.04455	1.02227	26.72	<0.001
	residual	32	1.22405	0.03825		
	Total	58	7.28369			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table H.6 ANOVA of F418 CFU/g DWS (log_{10}) in pasteurised soil at 22 % GWC with N, P, N+P or no fertiliser over 29 weeks at 15 °C in laboratory microcosms.

Source of variation		d.f.	s.s.	m.s.	F	P
Replicate.fertiliser stratum	replicate	2	0.03751	0.01875	0.86	0.471
	fertiliser	3	2.10141	0.70047	31.97	<0.001
	residual	6	0.13147	0.02191	1.23	
Replicate.fertiliser.time stratum	Time	7	3.33072	0.47582	26.71	<0.001
	Linear cpt	1	0.95568	0.95568	53.65	<0.001
	fertiliser.time	21	2.43316	0.11586	6.50	<0.001
	Linear cpt	3	2.00594	0.66865	37.54	<0.001
	residual	55	0.97968	0.01781		
	Total	94	9.00561			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table H.7 ANOVA of F418 and F418 *gfp* tr1 CFU/g DWS (log_{10}) in pasteurised and non-sterile soil at 22 % GWC with N, P, N+P or no fertiliser over 22 weeks at 15 °C in laboratory microcosms.

Source of variation		d.f.	s.s.	m.s.	F	P
Replicate.soil/fungus stratum	replicate	2	0.23155	0.11578	1.06	0.426
	Soil/fungus	2	15.10900	7.55450	69.40	<0.001
	residual	4	0.43543	0.10886	1.70	
Replicate.fertiliser stratum	fertiliser	3	1.73026	0.57675	12.06	0.002
	residual	8	0.38274	0.04784		
Replicate.soil/fungus.fertiliser stratum	Soil/fungus.fertiliser	6	1.62993	0.27166	4.24	0.022
	residual	10	0.64052	0.06405	2.46	
Replicate.fertiliser.time stratum	Time	6	47.83727	7.97288	307.62	<0.001
	Linear cpt	1	41.44094	41.44094	1598.93	<0.001
	Fertiliser.time	18	3.54527	0.19696	7.60	<0.001
	Linear cpt	3	2.98545	0.99515	38.40	<0.001
	residual	48	1.24406	0.02592	1.00	
Replicate.soil/fungus.fertiliser.time stratum	Soil/fungus.time	12	18.73808	1.56151	60.05	<0.001
	Linear cpt	2	17.62197	8.81099	338.87	<0.001
	Soil/fungus.fertiliser.time	36	5.20143	0.14448	5.56	<0.001
	Linear cpt	6	3.32958	0.55493	21.34	<0.001
	residual	86	2.23611	0.02600		
	total	241	75.01716			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Appendix I

SSCP reagents and gel

5 × TBE buffer

To make 1 L of 5 × TBE buffer, mix:

Tris base (J.T. Baker, USA)	54 g
Boric acid (BDH, Prolabo®, AnalaR®, NORMAPUR®, VWR, Belgium)	27.5 g
0.5 M EDTA at pH 8	20 mL

Add distilled water to make up 1 L.

MDE gel for SSCP

To make 40 mL of MDE gel, mix:

2 × MDE® gel solution (Lonza Rockland Inc., ME, USA)	12.5 mL
5 × TBE	8 mL
Distilled water	19.5 mL
TEMED Tetramethylethylenediamine (Bio-Rad Laboratories Inc., CA, USA)	30 µL
10 % ammonium persulfate(0.05 g/0.5 mL) (Bio-Rad Laboratories Inc., USA)	280 µL

MDE gel contains 31 % MDE® 2 ×, 20 % 5 × TBE, 0.075 % TEMED and 0.07 % ammonium persulfate

Fixer

Distilled water	900 mL
Ethanol 99 %	100 mL
Acetic acid (Fisher Scientific, UK)	5 mL

Silver stain

Fixer (described above)	250 mL
silver nitrate $AgNO_3$ (BDH, UK)	0.5 g

Developper

Distilled water	500 mL
sodium hydroxide NaOH (LabServ, Biolab, Australia)	15 g
formaldehyde CH_2O (Fisher Scientific, UK)	0.5 mL

Denaturing loading dye

30 µL of denaturing loading dye contains 95 % formamide (Fisher Scientific), 0.05 % bromophenol blue (BDH),0.0005 % xylene cyanol (BDH), 10 mM NaOH (LabServ), 0.8 mM EDTA pH8)

Appendix J

Mycelial growth diameter on membrane sandwich

Data for mycelial growth rings measurements were analysed using general ANOVA with factorial treatments structures and interactions. Only the size of mycelial rings from the membranes in pasteurised soil were statistically analysed as there was not enough mycelial rings on membranes in non-sterile soil to be analysed.

Table J.1 F418 *gfp* tr3 mycelial growth ring diameters on membranes sandwich in pasteurised and non-sterile soil at 22 and 31 % GWC

	Week 1	Week 2	Week 3	Week 4	Week 7	Week 10	Week 21
Pasteurised soil at 31 % WC	4.75	4.38	4.56	4.63	4.75	4.56	4.50
Pasteurised soil at 22 % WC	4.94	3.69	4.63	4.63	4.63	4.75	4.63
Non-sterile soil at 31 % WC					4.25	4.17	4.25
Non-sterile soil at 22 % WC				4.5	4.25	4	4.75
LSD (5 %)	**0.68**	**0.68**	**0.38**	**0.54**	**0.4**	**0.5**	**N/A**

Table J.2 F418 *gfp* tr3 mycelial growth ring diameters on membranes sandwich in pasteurised and non-sterile soil at -0.33 and -5 bar

Time (week)	0	1	2	4	8	12	16	20
Pasteurised soil -0.33 bar	5.95	5.13	5.46	5.20	5.21	5.42	4.73	4.77
Pasteurised soil -5 bar	5.95	5.69	5.49	5.68	5.58	5.52	5.29	5.49
Non-sterile soil -0.33 bar	5.95	5.99	6.00	5.94	5.90	5.88	5.52	5.16
Non-sterile soil -5 bar	5.95	5.90	5.83	5.77	5.78	5.66	5.64	5.72
LSD (5 %)		**0.29**	**0.29**	**0.36**	**0.38**	**0.30**	**0.37**	**0.54**

Appendix K
Statistical tables for Chapter 4

Table K.1 ANOVA of gravimetric moisture content (w/w) of non-sterile and pasteurised Templeton silt loam soil between -0.1 bar and -10 bar.

Source of variation	d.f.	s.s.	m.s.	F	P
replicate	2	0.166	0.083	0.04	0.961
soil	1	72.464	72.464	34.85	<.001
pressure	6	2372.026	395.338	190.11	<.001
Linear cpt	1	1505.426	1505.426	723.91	<.001
Soil.pressure	6	1.461	0.244	0.12	0.993
Linear cpt	1	0.127	0.127	0.06	0.807
residual	26	54.069	2.080		
Total	41	2500.186			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table K.2 ANOVA of F418 *gfp* tr3 CFU/g DWS (log_{10}) in pasteurised and non-sterile soil at 22 and 31 % GWC over 10 weeks at 15 °C in laboratory microcosms

Source of variation		d.f.	s.s.	m.s.	F	P
Block.time stratum	block	3	0.08641	0.02880	1.54	0.235
	time	7	0.14875	0.02125	1.13	0.380
	Linear cpt	1	0.00096	0.00096	0.05	0.824
	residual	21	0.39388	0.01876	1.14	
Block.time.moisture.soil	moisture	1	1.62451	1.62451	99.07	<0.001
stratum	Linear cpt	1	1.62451	1.62451	99.07	<0.001
	soil	1	4.71894	4.71894	287.79	<0.001
	time.moisture	7	0.27809	0.03973	2.42	0.028
	Linear cpt	1	0.15810	0.15810	9.64	0.003
	time.soil	7	0.81228	0.11604	7.08	<0.001
	Linear cpt	1	0.65431	0.65431	39.90	<0.001
	moisture.soil	1	2.28920	2.28920	139.61	<0.001
	Linear cpt	1	2.28920	2.28920	139.61	<0.001
	time.moisture.soil	7	0.35122	0.05017	3.06	0.007
	Linear cpt	1	0.11818	0.11818	7.21	0.009
	residual	72	1.18060	0.01640		
	total	127	11.88387			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table K.3 ANOVA of F418 *gfp* tr3 CFU/g DWS (log_{10}) in pasteurised and non-sterile soil at -0.33 and -5 bar over 18 weeks at 15 °C in laboratory microcosms

Source of variation		d.f.	s.s.	m.s.	F	P
Block.time stratum	block	3	0.04590	0.01530	0.55	0.652
	time	7	0.38411	0.05487	1.98	0.106
	Linear cpt	1	0.26848	0.26848	9.71	0.005
	residual	21	0.58066	0.02765	1.93	<0.001
Block.time.soil.moisture	soil	1	2.28657	2.28657	159.88	<0.001
stratum	moisture	1	6.87346	6.87346	480.62	<0.001
	Linear cpt	1	6.87346	6.87346	480.62	<0.001
	time.soil	7	0.87996	0.12571	8.79	<0.001
	lin.soil	1	0.30408	0.30408	21.26	<0.001
	time.moisture	7	1.12091	0.16013	11.20	<0.001
	Linear cpt	1	0.39027	0.39027	27.29	<0.001
	soil.moisture	1	6.48011	6.48011	453.11	<0.001
	Linear cpt	1	6.48011	6.48011	453.11	<0.001
	time.soil.moisture	7	0.83316	0.11902	8.32	<0.001
	Linear cpt	1	0.27123	0.27123	18.97	<0.001
	residual	72	1.02970	0.01430		
	total	127	20.51454			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table K.4 ANOVA of F418 *gfp* tr3 washed propagules (log_{10}) in membrane sandwiches in pasteurised and non-sterile soil at 22 and 31 % GWC over 21 weeks at 15 °C in laboratory microcosms

Source of variation		d.f.	s.s.	m.s.	F	P
rep.time stratum	rep	3	0.07472	0.02491	0.53	0.669
	time	6	4.68116	0.78019	16.51	<0.001
	Linear cpt	1	0.57258	0.57258	12.12	0.003
	residual	18	0.85038	0.04724	1.07	
rep.time.soil.moisture	soil	1	0.57310	0.57310	12.92	<0.001
stratum	moisture	1	0.06480	0.06480	1.46	0.232
	Linear cpt	1	0.06480	0.06480	1.46	0.232
	time.soil	6	2.96283	0.49380	11.13	<0.001
	Linear cpt	1	0.02808	0.02808	0.63	0.429
	time.moisture	6	0.70428	0.11738	2.65	0.025
	Linear cpt	1	0.16672	0.16672	3.76	0.057
	Soil.moisture	1	0.52629	0.52629	11.87	0.001
	Linear cpt	1	0.52629	0.52629	11.87	0.001
	time.soil.moisture	6	1.28361	0.21394	4.82	<0.001
	Linear cpt	1	0.05277	0.05277	1.19	0.280
	residual	57	2.52820	0.04435		
	total	105	13.84279			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table K.5 ANOVA of F418 washed *gfp* tr3 propagules (log_{10}) from membranes sandwiches in pasteurised and non-sterile soil at -0.33 and -5 bar over 20 weeks at 15 °C in laboratory microcosms

Source of variation		d.f.	s.s.	m.s.	F	P
Block.time stratum	block	3	0.24839	0.08280	1.74	0.194
	time	6	3.02105	0.50351	10.59	<0.001
	Lin	1	2.48906	2.48906	52.34	<0.001
	residual	18	0.85599	0.04756	0.89	
Block.time.moisture.soil	moisture	1	1.07022	1.07022	19.95	<0.001
stratum	lin	1	1.07022	1.07022	19.95	<0.001
	soil	1	5.18228	5.18228	96.59	<0.001
	time.moisture	6	1.45711	0.24285	4.53	<0.001
	lin.lin	1	0.62327	0.62327	11.62	0.001
	time.soil	6	0.25024	0.04171	0.78	0.591
	lin.soil	1	0.03201	0.03201	0.60	0.443
	moisture.soil	1	1.22101	1.22101	22.76	<0.001
	Lin.soil	1	1.22101	1.22101	22.76	<0.001
	time.moisture.soil	6	0.23851	0.03975	0.74	0.619
	Lin.lin.soil	1	0.06628	0.06628	1.24	0.271
	residual	62	3.32647	0.05365		
	total	110	16.76423			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Appendix L

Statistical table for Chapter 5

Table L.1 ANOVA of F418 *gfp* tr3 CFU/g DWS (log_{10}) in pasteurised and non-sterile soil at 22 % GWC with host, non-host or no insect over 12 weeks at 15 °C in laboratory microcosms

Source of variation		d.f.	s.s.	m.s.	F	P
Block.time stratum	block	3	0.22166	0.07389	1.14	0.366
	Time	5	0.35872	0.07174	1.10	0.399
	Linear cpt	1	0.19729	0.19729	3.03	0.102
	residual	15	0.97538	0.06503	1.03	
Block.time.insect.soil	insect	2	3.10665	1.55333	24.51	<0.001
stratum	Soil	1	0.60839	0.60839	9.60	0.003
	Time.insect	10	2.89961	0.28996	4.58	<0.001
	Linear cpt	2	2.39005	1.19503	18.86	<0.001
	Time.soil	5	0.55438	0.11088	1.75	0.131
	Linear cpt	1	0.00079	0.00079	0.01	0.911
	Insect.soil	2	0.77713	0.38856	6.13	0.003
	Time.insect.soil	10	0.40904	0.04090	0.65	0.771
	Linear cpt	2	0.08229	0.04114	0.65	0.525
	residual	90	5.70411	0.06338		
	total	143	15.61506			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table L.2 ANOVA of F418 *gfp* tr3 CFU/g DWS (log_{10}) in pasteurised and non-sterile soil at -2 bar with host, non-host or no insect over 16 weeks at 15 °C in laboratory microcosms

Source of variation		d.f.	s.s.	m.s.	F	P
Block.time stratum	block	3	0.02049	0.00683	0.09	0.966
	Time	6	11.07624	1.84604	23.65	<0.001
	Linear cpt	1	10.51700	10.51700	134.75	<0.001
	residual	18	1.40485	0.07805	1.01	
Block.time.insect.soil	insect	2	14.80127	7.40064	95.49	<0.001
stratum	Soil	1	9.66207	9.66207	124.66	<0.001
	Time.insect	12	18.01291	1.50108	19.37	<0.001
	Linear cpt	2	17.05904	8.52952	110.05	<0.001
	Time.soil	6	3.96235	0.66039	8.52	<0.001
	Linear cpt	1	2.09019	2.09019	26.97	<0.001
	Insect.soil	2	1.18586	0.59293	7.65	<0.001
	Time.insect.soil	12	2.23001	0.18583	2.40	0.009
	Linear cpt	2	0.56728	0.28364	3.66	0.029
	residual	105	8.13802	0.07750		
	total	167	70.49407			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Appendix M

Statistical tables for Chapter 6

Table M.1 ANOVA of F418 *gfp* tr3 CFU/g DWS (log_{10}) in pasteurised and non-sterile soil at -0.5 bar with or without red clover plant over 18 weeks at 15 °C in laboratory microcosms

Source of variation		d.f.	s.s.	m.s.	F	P
rep.time stratum	rep	3	2.57137	0.85712	12.98	<0.001
	Time	6	4.45849	0.74308	11.26	<0.001
	Linear cpt	1	0.68679	0.68679	10.40	0.005
	residual	18	1.18817	0.06601	0.92	
rep.time.soil.plant	soil	1	35.24737	35.24737	490.87	<0.001
stratum	plant	1	0.01794	0.01794	0.25	0.619
	Time.soil	6	4.54892	0.75815	10.56	<0.001
	Linear cpt	1	1.64874	1.64874	22.96	<0.001
	Time.plant	6	0.04781	0.00797	0.11	0.995
	Linear cpt	1	0.00316	0.00316	0.04	0.834
	soil.plant	1	0.00041	0.00041	0.01	0.940
	Time.soil.plant	6	0.12010	0.02002	0.28	0.945
	Linear cpt	1	0.03926	0.03926	0.55	0.462
	residual	62	4.45196	0.07181		
	total	110	52.16169			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table M.2 ANOVA of F418 *gfp* tr3 CFU/g DWS (log_{10}) in pasteurised and non-sterile soil at -0.5 bar in the bulk or rhizosphere soil of red clover plant over 18 weeks at 15 °C in laboratory microcosms

Source of variation		d.f.	s.s.	m.s.	F	P
rep.time stratum	rep	3	1.7801	0.5934	5.01	0.011
	Time	6	6.6885	1.1148	9.40	<0.001
	Linear cpt	1	1.0514	1.0514	8.87	0.008
	residual	18	2.1335	0.1185	1.13	<0.001
rep.time.soil.type	soil	1	48.2230	48.2230	461.10	<0.001
stratum	type	1	1.3692	1.3692	13.09	<0.001
	Time.soil	6	7.1121	1.1854	11.33	<0.001
	Linear cpt	1	4.1784	4.1784	39.95	<0.001
	Time.type	6	1.0752	0.1792	1.71	0.208
	Linear cpt	1	0.1690	0.1690	1.62	0.140
	soil.type	1	1.3774	1.3774	13.17	0.064
	Time.soil.type	6	1.3275	0.2213	2.12	0.027
	Linear cpt	1	0.5336	0.5336	5.10	0.197
	residual	63	6.5887	0.1046		
	total	111	77.6754			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value

Table M.3 ANOVA of red clover plants weights (log_{10} mg) in pasteurised and non-sterile soil at -0.5 bar with or without F418 *gfp* tr3 over 18 weeks at 15 °C in laboratory microcosms

Source of variation		d.f.	s.s.	m.s.	F	P
rep.time stratum	rep	3	0.73871	0.24624	4.65	0.017
	time	5	52.48743	10.49749	198.27	<0.001
	Linear cpt	1	41.04398	41.04398	775.23	<0.001
	residual	15	0.79416	0.05294	1.03	
rep.time.fungus.soil	fungus	1	0.00023	0.00023	0.00	0.947
stratum	soil	1	0.07273	0.07273	1.41	0.240
	time.fungus	5	0.06486	0.01297	0.25	0.937
	Linear cpt	1	0.00003	0.00003	0.00	0.981
	time.soil	5	0.09791	0.01958	0.38	0.860
	Linear cpt	1	0.02086	0.02086	0.41	0.527
	fungus.soil	1	0.00387	0.00387	0.08	0.785
	time.fungus.soil	5	0.25423	0.05085	0.99	0.434
	Linear cpt	1	0.03226	0.03226	0.63	0.432
	residual	54	2.78033	0.05149		
	total	95	57.29445			

NB: d.f. = degrees of freedom; s.s = sum of the squares; m.s. = mean square; F = F ratio; P = P value